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Targeting Brd4 for cancer therapy: inhibitors and degraders

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Bromodomain-containing protein 4 (Brd4) plays an important role in mediating the expression of genes involved in cancers and non-cancer diseases such as inflammatory diseases and acute heart failure. Inactivating Brd4 or downregulating its expression inhibits cancer development, leading to the current interest in Brd4 as a promising anticancer drug target. Numerous Brd4 inhibitors have been studied in recent years and some of them are currently in various phases of clinical trials. Recently, selective degradation of target proteins by small bifunctional molecules (PROTACs) has emerged as an attractive drug discovery approach owing to the advantages it could offer over traditional small-molecule inhibitors. A number of Brd4 degraders have been reported and showed more efficient anticancer activities than just protein inhibition. In this review, we will discuss recent findings in the discovery and development of small-molecule inhibitors and degraders that target Brd4 as a potential anticancer agent.

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Introduction

Reversible lysine acetylation plays an essential role in the epigenetic regulation of chromatin structure and transcription of genes *via* modification of histone proteins and transcription factors. These changes in gene expression are modulated by three categories of epigenetic regulatory proteins, which are commonly known as “writers”, “erasers”, and “readers”. Histone acetyltransferases (HATs) function as “writers” to acetylate lysine residues on histone tails,¹ while histone deacetylases (HDACs) act as “erasers” to remove the acetyl group from acetylated lysine (Kac).^{2,3} The third kind of epigenetic regulatory protein is the bromodomain family of proteins that selectively bind to acetylated lysines, thus functioning as “readers” of the lysine acetylation state.⁴

The bromodomain and extra-terminal (BET) family is a subset of 46 bromodomain-containing proteins found only in the human genome.⁵ BET proteins are composed of four proteins, namely bromodomain-containing protein 2 (Brd2), Brd3, Brd4 and bromodomain testis-specific protein (BrdT).⁶ Each BET family member contains tandem N-terminal bromodomains (BD1 and BD2) and an extra C-terminal domain (ET) exhibiting high levels of sequence conservation.⁷

Each bromodomain of Brd4 shares a conserved fold comprising a left-hand bundle of four antiparallel α helices (α A, α B, α C and α Z) linked by two hydrophobic loop structures, namely the ZA loop formed between the α Z and α A helices and the BC loop formed between the α B and α C helices. The loop regions of Brd4 BD1 (Brd4(1)) and BD2 (Brd4(2)) diverge slightly in sequence and length which contributes to acetyl-lysine binding specificity.⁸ Cocrystal structures between Brd4(1) and peptidic substrates show that helices α B and α C and the ZA loop form a hydrophobic Kac-recognition pocket at the helical bundle terminus. The top of the pocket usually contains a well-conserved asparagine residue 140 (Asn140), which engages in a direct hydrogen bonding to the Kac. Simultaneously, a second hydrogen bond is formed between an acetyl carbonyl oxygen atom and the phenol of a conserved Tyr97 *via* a structured water molecule. A conserved hydrophobic region, the WPF shelf, formed among the ZA loop, BC loop and α Z, is also important for Brd4 binding affinities.^{6,9} Brd4 regulates gene expression through its ability to bind to Kac residues of histone tails, followed by recruiting the positive transcription elongation factor b (P-TEFb) to phosphorylate RNA polymerase II (RNA Pol II).¹⁰

Deregulation of BET proteins, in particular Brd4, has been implicated in the development of diverse diseases, especially cancers.¹¹ Zuber *et al.* demonstrated that Brd4 played an important role in maintaining c-Myc expression to promote aberrant self-renewal of AML cells. Knockdown of Brd4 using shRNAs or pharmacologic inhibition of Brd4 with a small-molecule inhibitor resulted in induction of terminal differentiation and elimination of leukemia stem cells and showed

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potent anti-leukemic effects in a variety of human AML cell lines and primary patient-derived cells.^{12,13} Hunter and colleagues identified *Brd4* as an inherited susceptibility gene that robustly predicted progression, metastasis and survival for breast cancer. *Brd4* dynamically regulated breast cancer metastasis through modulation of the extracellular matrix gene expression.^{14,15} Malley *et al.* demonstrated that BET protein inhibition potently suppressed the growth of tamoxifen-resistant breast cancer cells. Co-treatment with BET inhibitor and the ER degrader fulvestrant in a tamoxifen-resistant breast cancer xenograft mouse model displayed a strong long-lasting anticancer effect.¹⁶ In NSCLC tissues and NSCLC cell lines with higher invasion and metastasis potentials, *Brd4* expression was significantly up-regulated. Suppression of *Brd4* expression in NSCLC cell lines impaired cell invasion, inhibited cell proliferation, and accelerated cell apoptosis. More importantly, a high level of *Brd4* was closely correlated with the poor prognosis of NSCLC patients.¹⁷ *Brd4* was highly over-expressed in primary and metastatic melanoma tissues and essential for melanoma tumor growth. Treatment with *Brd4* inhibitors rapidly down-regulated key cell-cycle genes, including *SKP2*, *ERK1* and *c-Myc*, and strongly attenuated melanoma cell proliferation *in vitro* and tumor growth and metastatic behavior *in vivo*. Individual silencing of *Brd4* mostly recapitulated the potent anti-leukemic effects of *Brd4* inhibitor-mediated suppression. Notably, *Brd4* inhibitor treatment remained robustly effective against *BRAF* or *NRAS* mutant melanoma cells.¹⁸ Ayad *et al.* demonstrated that *Brd2* and *Brd4* were significantly elevated in glioblastoma (GBM). Depletion of *Brd4* using siRNA in GBM cells significantly reduced GBM cell proliferation by arresting cell cycle progression at the G₁/S phase. Similarly, treatment with the BET protein inhibitor I-BET151 inhibited GBM cell proliferation *in vitro* and *in vivo*.¹⁹ In castration-resistant prostate cancer (CRPC), knockdown of *Brd2*, 3 and 4 led to significant inhibition of cell proliferation and invasion, phenocopying the BET bromodomain inhibition treatment. In CRPC xenograft mouse models, BET inhibitors showed powerful anticancer activity and were more efficacious than direct AR antagonists.²⁰

In view of the intimate link between *Brd4* expression and cancers, *Brd4* has been considered as a promising therapeutic target in many malignancies.^{21,22} Significant efforts have been made to develop pharmacological inhibitors of *Brd4*, and a number of *Brd4* inhibitors have progressed to clinical and preclinical evaluation.^{23,24} Recently, *Brd4* degraders based on protein proteolysis-targeting chimera (PROTAC) technology have emerged as a novel approach for the epigenetic therapy of cancers and displayed exciting antitumor efficiency. Herein, we mainly focus on discussing recent advances in small-molecule inhibitors and degraders of *Brd4* developed for cancer therapy.

1 *Brd4* inhibitors

Since the first *Brd4* inhibitor (+)-JQ1 was developed in 2010, numerous *Brd4* inhibitors have been discovered in the past few years. According to the interaction mode between BDs

and inhibitors, there are two classes of *Brd4* inhibitors: monovalent and bivalent. Monovalent *Brd4* inhibitors bind to each bromodomain of *Brd4* protein separately. In contrast, bivalent *Brd4* inhibitors are capable of engaging both bromodomains simultaneously within *Brd4*. The most widely studied *Brd4* inhibitors are monovalent *Brd4* inhibitors, which are mainly divided into the following eight categories according to the similarity of their chemical structures: triazoloazepine derivatives, isoxazole derivatives, pyridine derivatives, tetrahydroquinoline derivatives, triazolopyrazine derivatives, 4-acyl pyrrole derivatives, 2-thiazolidinone derivatives and others (Table 1). Moreover, several *Brd4* inhibitors with disclosed structure have been enrolled into different phases of human clinical trials.²⁵

1.1 Monovalent *Brd4* inhibitors

1.1.1 Triazoloazepine-based *Brd4* inhibitors. Inspired by the observation from Mitsubishi Pharmaceuticals that simple thienodiazepines possessed binding activity for *Brd4*, Bradner *et al.* reported the first potent and selective BET protein inhibitor (+)-JQ1 (1) containing a thieno-triazolo-1,4-diazepine scaffold (Fig. 1). The co-crystal structure showed that (+)-JQ1 entirely occupied the acetyl-lysine binding pocket by forming a hydrogen bond between the methyltriazole moiety and the conserved asparagine140. (+)-JQ1 strongly inhibited *Brd4*(1) with an IC₅₀ value of 77 nM and a K_d value of 50 nM, determined by alpha-screen and isothermal titration calorimetry (ITC) assays, respectively. In contrast, its enantiomer (-)-JQ1 showed no significant interaction with any BET proteins. In several NUT midline carcinoma xenograft models, remarkable tumor regression and prolonged overall survival were observed after treatment with (+)-JQ1 for 18 days at a well-tolerated dose. (+)-JQ1 also showed robust antitumor efficacy in several other xenograft models including multiple myeloma and AML.^{11,26} The development of (+)-JQ1 revealed novel insights into the therapeutic potential of inhibiting *Brd4* and aroused an upsurge of research on *Brd4* inhibitors.

Exploring the effect of the substituent on the chain moiety of (+)-JQ1 led to a number of thienodiazepine-based *Brd4* inhibitors. The representative compounds 2–6 are shown in Fig. 1. Among them, the two best known are OTX015 (2)^{27–29} and TEN-010 (3),³⁰ which have entered a phase I clinical trial for the treatment of hematological malignancies and other cancers. Compound 4, patented by Tensha Therapeutics,³¹ and compound 5, patented by Bayer,³² exhibited potent inhibition against *Brd4*(1) with an IC₅₀ value of 0.43 nM and 27 nM, respectively. Compound 6 was generated from a phenotypic drug discovery study on thienodiazepine derivatives and showed impressive *Brd4*(1) inhibitory activity with an IC₅₀ value of 34 nM. In addition to robust *in vivo* antitumor efficacy with a tumor growth inhibition (TGI) of 80% at a dose of 10 mg kg⁻¹ twice a day, compound 6 also displayed potent immunosuppressive activity in a mouse collagen-induced arthritis model.³³

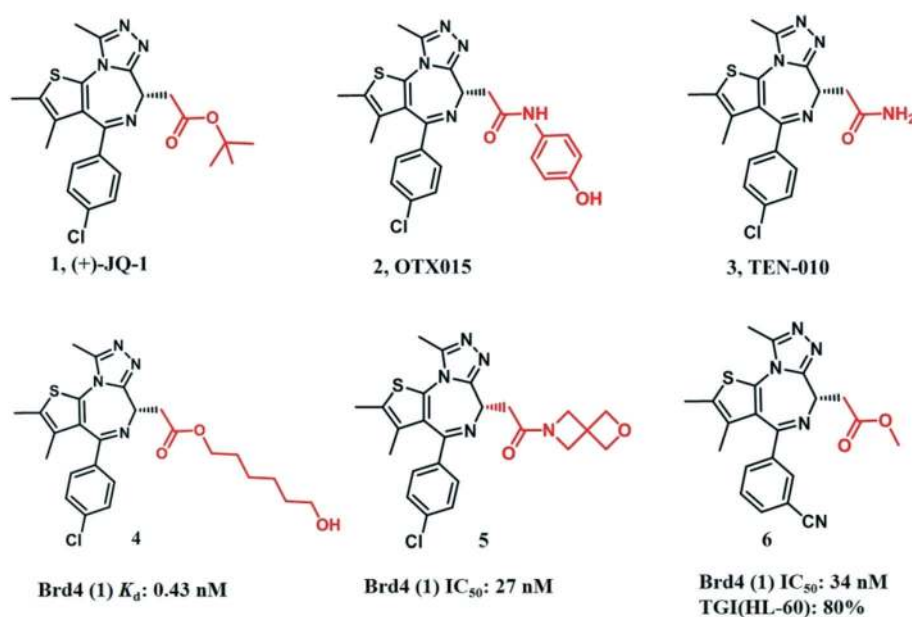
Table 1 Overview of small-molecule Brd4 inhibitors

	Structure features	Compounds	Compounds in clinical trials	Clinical phase	NCT identifier	Ref.		
Monovalent Brd4 inhibitors	Triazoloazepines	1–15	2 (OTX015)	I	NCT02698189 NCT02698176 NCT01713582 NCT02259114	27–29, 128		
			3 (TEN-010) 10 (GSK525762, I-BET762)	I I/II	NCT02308761 NCT01943851 NCT01587703 NCT02964507 NCT01949883	30 20, 35, 36 41		
		Isoxazoles	16–42	19 (CPI-0610)	I	NCT02157636 NCT02158858	47, 48 53	
				28 (I-BET151)	I	NCT02630251	55	
				36 (PLX51107)	I	NCT02683395	60, 61	
				38 (INCB0543294)	I/II	NCT02431260		
				46 (ABBV-075)	I	NCT02391480		
				BI 894999 ^a	I	NCT02516553		
		Pyridines	43–55	43–55	46 (ABBV-075)	I	NCT02391480	86
Tetrahydroquinolines	56–62	63–69	BI 894999 ^a	I	NCT02516553	94, 95		
Triazolopyrazines	63–69	70–74	82 (BMS-986158)	I/II	NCT02419417	86		
4-Acyl pyrroles	70–74	75–77	89 (AZD5153)	I	NCT03205176	94, 95		
Thiazolidinones	75–77	78–85	89 (AZD5153)	I	NCT03205176	94, 95		
Others	78–85	86–89	89 (AZD5153)	I	NCT03205176	94, 95		
Bivalent Brd4 inhibitors	Triazolopyridazines	86–89	89 (AZD5153)	I	NCT03205176	94, 95		
	Triazoloazepines	90						

^a Specific chemical structure is not disclosed.

A parallel but independent study from GlaxoSmithKline described a series of compounds with different substitutions on benzodiazepine as potent Brd4 inhibitors (Fig. 2).³⁴ Compound 7 was initially developed as an ApoA1 upregulator with an EC₁₇₀ of 0.22 μM (EC₁₇₀ is the concentration of compound resulting in a 70% increase in luciferase activity). However, subsequent research studies confirmed that the ApoA1 up-regulation was caused by directly targeting BET proteins with a pIC₅₀ of 6.3 for Brd4. Various chemical modifications on

compound 7 were carried out to explore more potent BET inhibitors. Compound 8 with potent Brd4 inhibition (pIC₅₀ of 6.4) was generated; however, the remarkable Brd4 inhibition potency was counteracted by the fact that compound 8 was prone to undergo a ring-opening and hydrolysis reaction in an acidic aqueous solution ($T_{1/2}$ = 0.23 h at pH 2). It was found that compound 8 bound to Brds in a highly stereospecific manner, as exemplified by the (*R*)-enantiomer (+)-8, which is 251 times more potent than the (*S*)-enantiomer

**Fig. 1** Reported thienotriazolodiazepine-based Brd4 inhibitors.

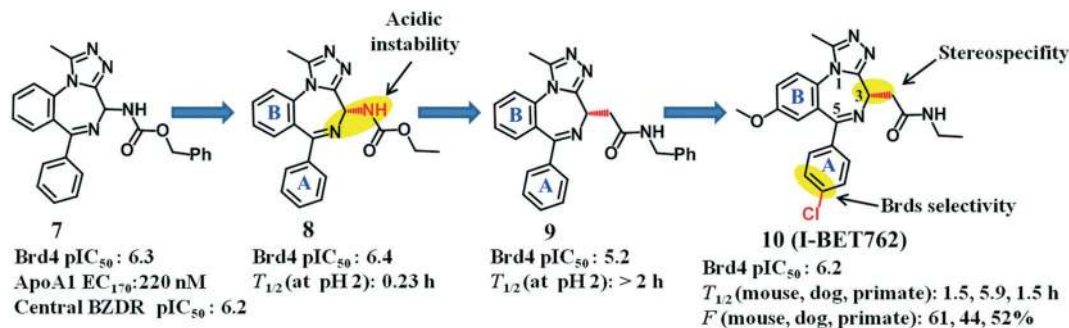


Fig. 2 Discovery and development of Brd4 inhibitor I-BET762.

against Brd4. Compound 9 with improved acidic stability ($T_{1/2} > 2$ h at pH 2) was achieved by changing the carbamate group of compound 8 to an amide, but a 10-fold decrease in inhibition for Brd4 was observed.

Further modification of acid-stable compound 9 was focused on improving its potency and physicochemical properties. Substitution at the *p*- or *m*-position of ring A abolished the off-target GABA receptor activity, which was derived from the 1,4-benzodiazepine motif found in many marketed GABA receptor-positive allosteric modulators. Higher potency and better drug-like properties were obtained by introducing a methoxyl substituent at the 8 position of the B ring and replacing the benzyl group with alkyl groups. All the favored modifications were incorporated into compound 9 and created a more druggable potent Brd4 inhibitor, compound 10 (namely I-BET762 or GSK525762), with excellent physicochemical and pharmacokinetic properties. I-BET762

displayed enhanced metabolic stability, good on-target Brd4 activity, excellent solubility, good tissue distribution, and good oral bioavailability (44–61% in mice, dogs, and primates). Potent anticancer activity was observed for I-BET762 as an oral agent in several xenograft models.^{20,35} I-BET762 is currently under evaluation in a phase I/II clinical trial for the treatment of different cancers.³⁶

By replacing the amide at the 3-position of I-BET762 with 1,3,4-oxadiazole, Bayer disclosed compound 11 (Fig. 3).³⁷ Compound 11 showed potent anti-Brd4(1) activity ($IC_{50} = 20$ nM) and was very active against MOLM-13 cancer cells ($EC_{50} = 140$ nM). Bayer also filed a patent for compounds 12 and 13, which were featured with triazolopyrazolodiazapine and triazolopyrrolodiazapine scaffolds, respectively.³⁸ Compounds 12 and 13 were potent against Brd4(1) ($IC_{50} = 140$ and 20 nM, respectively) and showed marked growth inhibition in multiple cell lines. Interestingly, researchers from Bayer found that

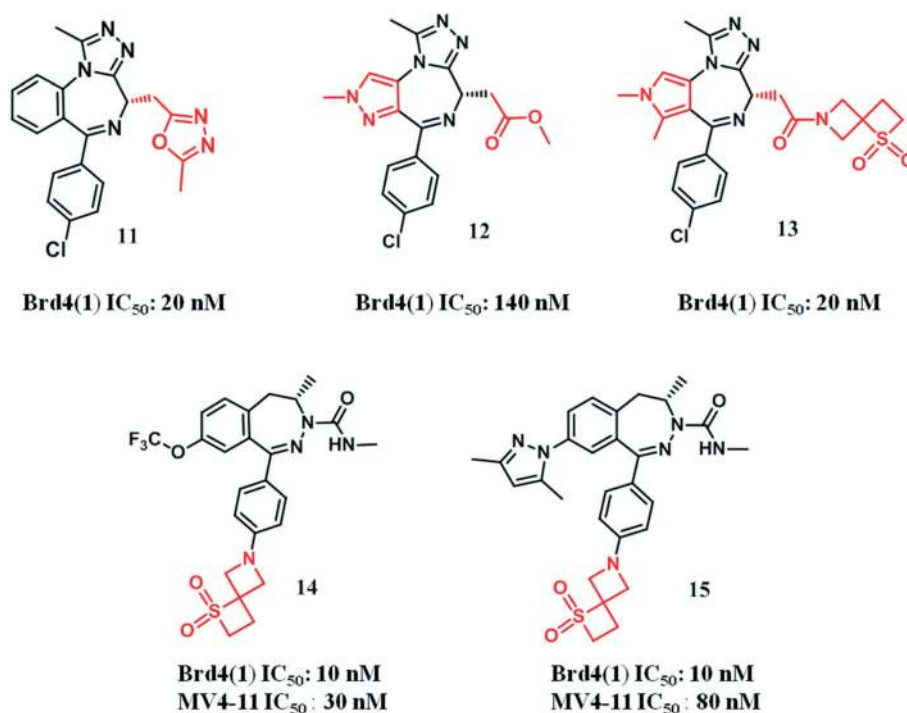


Fig. 3 Chemical structures of compounds 11–15.

isomeric benzodiazepine analogs without triazoles retained potency against Brd4.³⁹ Two representative compounds, 14 and 15, demonstrated the same potent Brd4(1) inhibition with IC₅₀ values of 10 nM and inhibited the growth of MV4-11 cancer cells with IC₅₀ values less than 100 nM.

1.1.2 Isoxazole-based Brd4 inhibitors. A fragment screen study from Albrecht *et al.* identified amino-isoxazole 16 as a weak Brd4(1) inhibitor with an IC₅₀ of 33 μM. The cocrystal structure of Brd4(1) with compound 16 revealed that compound 16 could bind to Brd4(1) in a mode similar to that of (+)-JQ1. Inspired by this finding, novel isoxazole azepine compound 17 was developed by hybridizing the isoxazole motif with an azepine scaffold, which displayed potent Brd4(1) inhibition (IC₅₀ = 290 nM) and moderate c-Myc suppression activity in Raji cells (IC₅₀ = 2.1 μM) (Fig. 4).⁴⁰ Further modification of this compound led to 18, which contained an amino group in place of the *tert*-butoxy group in compound 17.⁴¹ Compound 18 demonstrated around 10-fold better potency than compound 17 against Brd4(1) (IC₅₀ = 26 nM) and c-Myc (IC₅₀ = 140 nM). Compound 18 exhibited favorable pharmacokinetic profiles in rats with a *T*_{1/2} of 1.4 h and a bioavailability of 31%. P.O. dosing with compound 18 (10, 30, and 100 mg kg⁻¹) dose-dependently inhibited c-Myc mRNA expression *in vivo*, with an up to 75% reduction in c-Myc levels in the tumor at 4 h after a 100 mg kg⁻¹ dose. With the goal of avoiding the potential issue of metabolic instability, the thiophene ring in 18 was replaced with a more metabolically stable phenyl ring, and clinical candidate compound 19 (CPI-0610) was gained.⁴¹ Compound 19 was proved to have similar potency to 18 against Brd4 and more desirable pharmacokinetic parameters. *In vivo*, oral treatment of an MV4-11 xeno-

graft model with compound 19 caused substantial suppression of tumor growth with a maximum of 80% TGI at a dose of 30 mg kg⁻¹ twice daily and showed no obvious body weight loss. In the same model, co-treatment with compound 19 (10 mg kg⁻¹ subcutaneously twice daily) and doxorubicin (2 mg kg⁻¹ intravenously twice weekly) for 28 days resulted in complete inhibition of tumor growth. In patients with heavily pretreated diffuse large B-cell lymphoma and follicular lymphoma, treatment with compound 19 led to meaningful anti-tumor effects. Introducing an acetamide-substituted pyrazole to the 8-position of compound 19 produced compound 20.⁴² Compound 20 was potent against Brd4(1) (IC₅₀ = 17 nM) and c-Myc in MV4-11 cells (IC₅₀ = 32 nM). In comparison to compound 19, compound 20 showed a longer half-life and greater bioavailability. In MV4-11 tumor xenografts in Balb/c nude mouse models, compound 20 effectively attenuated c-Myc mRNA levels at doses of 5 (50% reduction) and 15 mg kg⁻¹ (75% reduction) twice a day.

A parallel but separate work from Heightman's group disclosed the 3,5-dimethylisoxazole motif as a Brd4 inhibitor.⁴³ It was observed that compound 20 bound in the acetylated-lysine recognition pocket of the Brd4(1) by forming a key hydrogen bond between the oxygen of the dimethylisoxazole and NH₂ of the conserved Asn 140 residue. Moreover, the dimethylisoxazole nitrogen atom interacts with the phenol group of Tyr-97 *via* the structured water molecule. The ethyl ether binds within the ZA channel and the methyl group attached to the secondary alcohol binds within the WPF shelf.

Replacing the methyl group with larger substituents such as aromatic rings to enhance the WPF shelf binding gave compounds 21 and 22. As expected, compared to compound

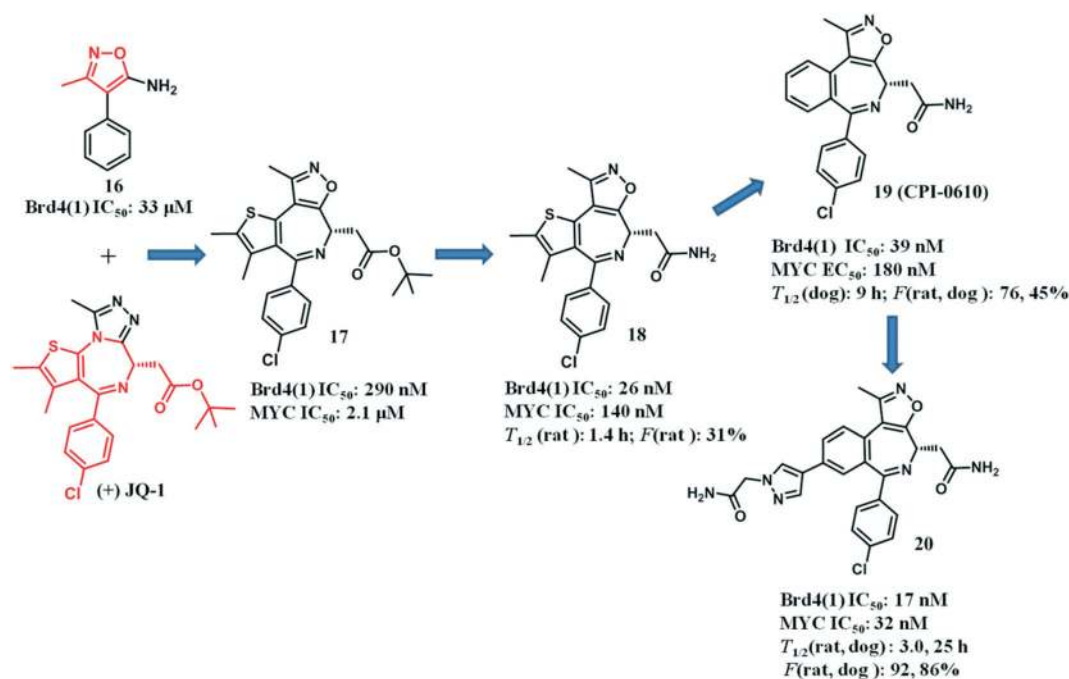


Fig. 4 Discovery and development of Brd4 inhibitor CPI-0610.

20, both 21 and 22 had significant improvement in Brd4 binding affinity with IC_{50} values of 382 and 371 nM, respectively.⁴⁴

Researchers from GSK developed compound 23 through structure-guided rational optimization of the privileged dimethylisoxazole chemotype.⁴⁵ Compound 23 had an IC_{50} value of 0.5 μ M against Brd4 and showed good anti-inflammatory activity in cellular assays, but poor solubility was observed. With the goal to improve the solubility, introduction of a polar group to the phenyl ring *para* to the isoxazole of 23 led to compound 24, which had a slightly decreased potency (IC_{50} = 2.6 μ M) but remarkable improved solubility as compared to compound 23.

Compound 25 was achieved using fluororous-tagged multi-component reactions and showed robust Brd4 binding ability with a K_d of 550 nM and cellular potency (IC_{50} = 724 nM) in Brd4-dependent lines.⁴⁶ Notably, compound 25 potently bound to two other bromodomain-containing proteins including TAF1 (IC_{50} = 560 nM) and TAF1L (IC_{50} = 1.3 μ M) (Fig. 5).

Most studies on 3,5-dimethylisoxazole-based Brd4 inhibitors have focused on introducing various heteroaromatic scaffolds to the 4-position of isoxazole.

Compound 26, which was initially developed as an ApoA1 up-regulator by utilizing a high-throughput screen approach, was proved to be a Brd4 inhibitor by researchers at GSK (Fig. 6).^{47,48} Optimizations of 26 to compound 27 created a more potent Brd4 inhibitor with an IC_{50} of 794 nM but as well frustrating potent CYP2C9 and CYP3A4 inhibition (IC_{50} = 2.8 and 3.9 μ M, respectively). Improvement of selectivity over CYP450 was achieved by eliminating $CONH_2$ and freezing the intramolecular hydrogen bond between C3 and C4 through a cyclization strategy to create a series of imidazolone analogues. Among them, I-BET151 (28) was the most promising Brd4 inhibitor (IC_{50} = 794 nM) with low CYP2C9 and CYP3A4 inhibition (IC_{50} = 9.9 and 9.7 μ M, respectively) and favorable PK properties suitable for oral exposure. I-BET151 demon-

strated remarkable potency in two distinct mouse models of murine MLL-AF9 and human MLL-AF4 leukemia.

Compounds 29–31 with tricyclic scaffolds similar to that of I-BET151 were reported as potent Brd4 inhibitors in a patent from Trillium Therapeutics, with IC_{50} values in the range of 10–21 nM.⁴⁹ It was found that the most potent compound 31 (IC_{50} = 10 nM) was quite unstable in both mouse and human liver microsomes due to the active CH_2 position which was prone to metabolism.

With the goal of expanding the chemical diversity of Brd4 inhibitors and enhancing the solubility of previously developed isoxazoloquinoline Brd4 inhibitors (27, 28), Mirguet *et al.* synthesized a series of 1,5-naphthyridine derivatives by replacing the benzene ring of compound 20 with a naphthyridine system.⁵⁰ Among them, compounds 32 and 33 showed the best pIC_{50} values for Brd4 (6.5 and 6.8, respectively) as well as good oral availability and solubility. Compounds 32 and 33 also showed efficacy in an acute inflammatory BALB/c mouse model.

Wang *et al.* reported a series of γ -carboline-containing compounds as novel small-molecule BET inhibitors (Fig. 6).⁵¹ The most potent inhibitor compound 34 bound to Brd2–4 proteins with K_i values of 3.2–24.7 nM as well as CREBBP protein with a K_d of 670 nM and showed good specificity over eight other non-BET bromodomain-containing proteins (K_d values \geq 10 000 nM). Notably, compound 34 demonstrated remarkable cell growth inhibition activity and excellent cellular specificity in AML cell lines. Compound 34 potently inhibited the viability of MV4-11 and MOLM-13 cells containing the MLL1 fusion gene, with IC_{50} values of 20 and 66 nM, respectively, while it showed no obvious inhibitory potency (IC_{50} > 2 μ M) against the K562 cell line harboring a Bcr-Abl fusion protein.

Recently, Wang's group developed novel BET inhibitors containing a 9H-pyrimido[4,5-*b*]indole tricyclic core structure by inserting one extra nitrogen atom into the 5H-pyrido[4,3-

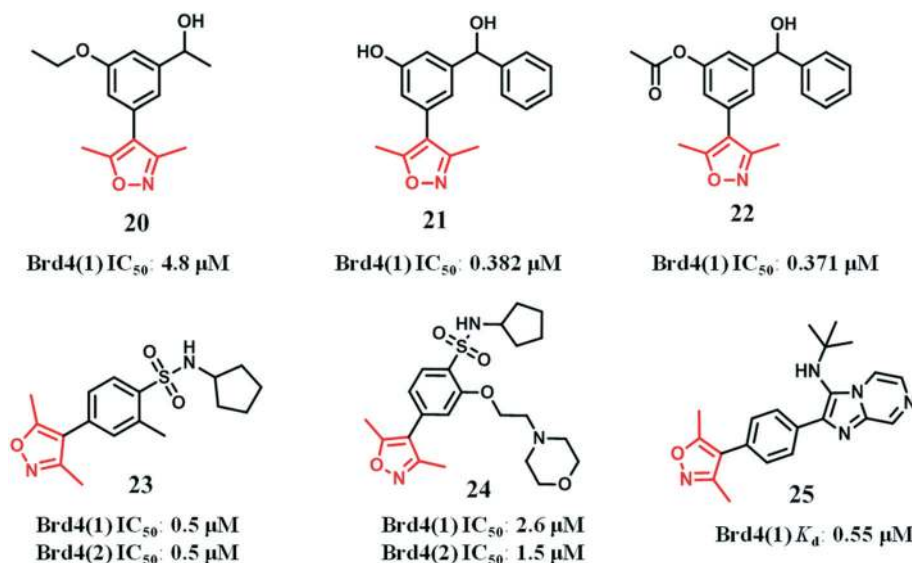


Fig. 5 Chemical structures of compounds 20–25.

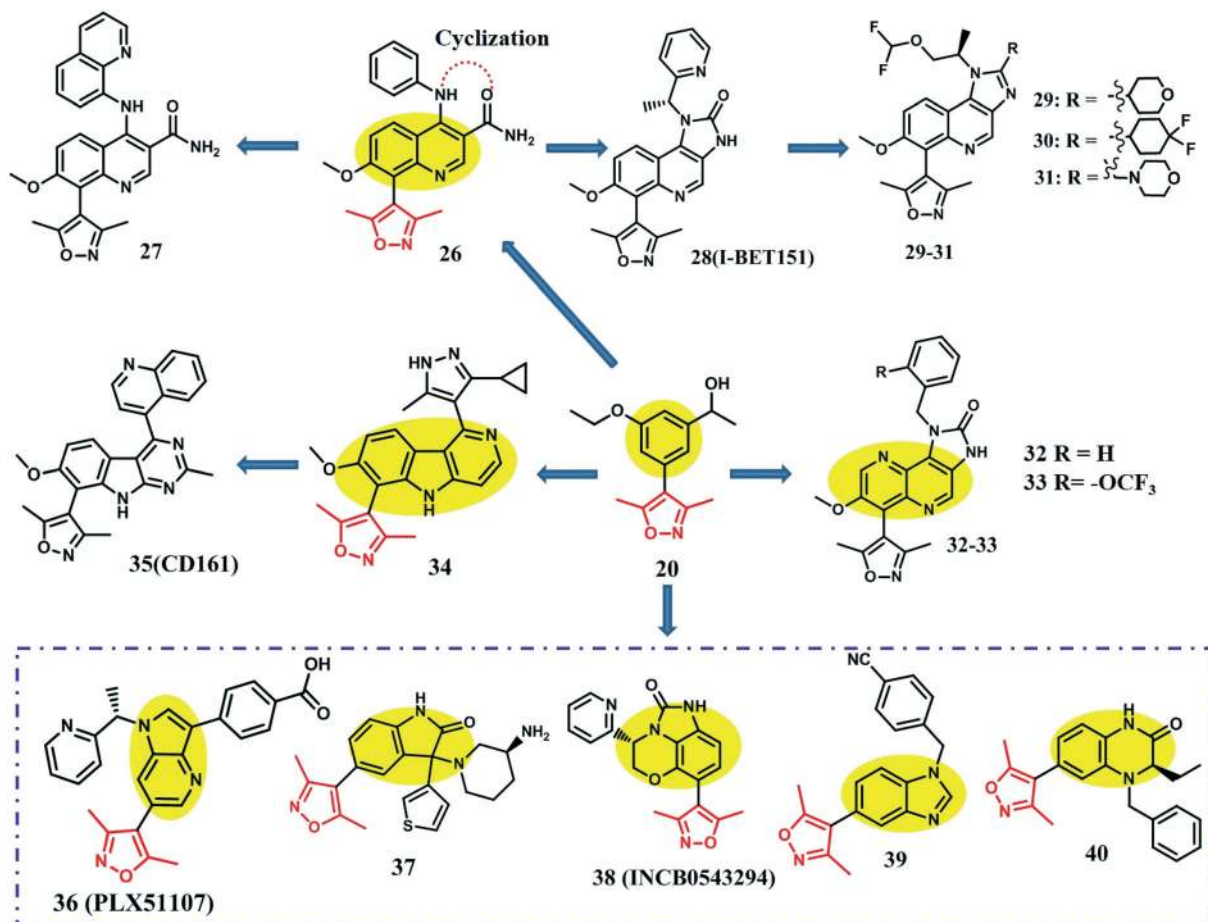


Fig. 6 Reported isoxazole-based Brd4 inhibitors.

h]indole of compound 34.⁵² A systematic SAR study led to the discovery of compound 35, which bound to Brd4(1) and Brd4(2) with K_i values of 8.2 nM and 1.4 nM, respectively. Compound 35 showed significant activities in inducing the down-regulation of Brd4-downstream *c-Myc* gene and up-regulation of p21 related to apoptosis in MV4-11 leukemia cells. This excellent Brd4 inhibition of compound 35 translated into dramatic antiproliferative effects against MV4-11 and MOLM-13 cell lines with IC_{50} values of 26 and 53 nM, respectively. In addition to acute leukemia cell lines, compound 35 also potently inhibited cell growth in nine breast cancer cell lines with IC_{50} values of <1 μ M. Compound 35 was found to have good solubility, microsomal stability in human microsomes and excellent oral bioavailability (93% in rat). In *in vivo* experiments, daily oral administration with compound 35 led to >80% and complete tumor regression in the MV4-11 and MDA-MB-231 mouse xenograft model, respectively, without inducing any toxicity or decline in weight gain.

Hatice *et al.* described compound 36 (PLX51107) to be a potent Brd4 inhibitor. PLX51107 potently bound to Brd4(1) and Brd4(2) with K_d values of 1.7 nM and 6.1 nM, respectively.⁵³ PLX51107 could significantly suppress the CpG-induced proliferation of primary chronic lymphocytic leukemia (CLL) cells with IC_{50} values in the range of 0.156 μ M to 10

μ M. In a *de novo* mouse model of CLL, PLX51107 significantly reduced leukemic disease burden in peripheral blood and spleen at 20 mg kg^{-1} (oral daily) and was more effective than 50 mg kg^{-1} OTX015. PLX51107 is currently in phase I clinical studies for solid tumor, AML and myelodysplastic syndrome.

BeiGene Ltd. has patented a series of substituted 5-(3,5-dimethylisoxazol-4-yl)indoline-2-one compounds including compound 37 as novel Brd4 inhibitors. Compound 37 inhibited Brd4 with an IC_{50} value of 8 nM in a TR-FRET methodology and showed promising antiproliferation cellular activity against the MV4-11 cell line (IC_{50} = 34 nM).⁵⁴

Phillip *et al.* described the preclinical activity of a novel Brd4 inhibitor compound 38 (INCB054329) with low nanomolar potency against Brd4 for the potential treatment of malignant diseases.⁵⁵ In both AML and lymphoma cell lines, INCB054329 remarkably inhibited expression of *c-Myc*, induced apoptosis consistent with increased expression of pro-apoptotic regulators, and suppressed cell growth with potencies of less than 200 nM. Oral administration of INCB054329 showed potent *in vivo* antitumor efficiency in several models of hematologic cancers. At present, INCB054329 is being tested in patients with any advanced solid malignancy or lymphoma by Incyte.

In pursuit of a potent and rapidly accessible Brd4 inhibitor, Brennan's group developed benzimidazole-substituted

3,5-dimethylisoxazoles as novel Brd4 inhibitors, which could be easily gained by three-step regioselective synthesis.⁵⁶ The most potent compound, 39, had a Brd4(1) IC₅₀ of 180 nM and showed marked selectivity for CREBBP as well as seven other tested bromodomain-containing proteins.

Yang *et al.* reported compound 40 as a potent Brd4 inhibitor by replacing the benzo[*d*]imidazole core of compound 39 with a dihydroquinoxalinone skeleton.⁵⁷ This modification conferred an extra interplay with the ZA channel region and a stronger interaction with the WPF shelf, which were reflected in superior Brd4 inhibition activity (IC₅₀ = 70 nM) and excellent antiproliferative activity in MV4-11 cancer cells (IC₅₀ = 258 nM) of compound 40.

More recently, Xu's group described a series of benzo[*d*]isoxazole-containing compounds as potent Brd4 inhibitors.⁵⁸ Among them, compounds 41 and 42 showed the highest binding affinities to Brd4(1) with K_d values of 82 and 81 nM, respectively (Fig. 7). Thermal stability shift assay and BROMOscan revealed that compounds 41 and 42 exhibited excellent selectivity over other non-BET subfamily members. Compounds 41 and 42 demonstrated a good inhibitory effect against acute leukemia cell lines (IC₅₀: 0.4–0.68 μM) and AR-positive prostate cancer cell lines (IC₅₀: 0.29–2.62 μM), while they showed weak cytotoxicity in the AR-negative prostate cancer cells and normal lung fibroblast cell line HFL-1. Consistent with the cell viability assays, compounds 41 and 42 significantly inhibited the colony formation of C4-2B and 22Rv1 cells at 0.5 μM. In a C4-2B CRPC xenograft tumor model treated intraperitoneally five times a week with compounds 41 and 42 for 25 days at a dose of 50 mg kg⁻¹, TGI values of 70% and 51% were achieved, respectively.

1.1.3 Pyridone-based Brd4 inhibitors. Researchers at AbbVie identified a novel phenylpyridazinone fragment, com-

pound 43, as a weak Brd4 binder (K_i = 160 μM) by a two-dimensional NMR fragment screen.⁵⁹ Compound 43 occupied the acetylated lysine binding pocket of Brd4(2) through two key interactions: hydrogen bonding of pyridone carbonyl oxygen to the amino group of conserved Asn433 and pyridone *N*-methyl substituent in the amphoteric pocket. Sustained efforts to achieve structural optimization of the privileged phenylpyridazinone moiety led to the discovery of a number of potent Brd4 inhibitors.

Compound 44 was found to potently bind to Brd4 with a K_i value of 13 nM and exhibited significant antiproliferative activity against MX-1 cancer cells with an EC₅₀ of 47 nM. A mouse study showed that an OPM-2 mouse xenograft model, dosed orally Q.D. with compound 44 for 21 days consecutively at doses of 1 mg kg⁻¹ and 3 mg kg⁻¹, demonstrated a TGI of 66% and 73%, respectively. Further chemical modification led to the macrocycle compound 45, which showed superior potency than compound 44 both in the cellular assay and *in vivo* assay.⁵⁹

By fusing a pyrrole ring with the pyridine core of compound 44, McDaniel *et al.* described a series of novel Brd4 inhibitors featured with a pyrrolopyridone scaffold, which could bidentately interact with the conserved asparagine residue of the Brd4 protein, as exemplified by the clinical candidate compound 46 (ABBV-075).⁶⁰ Compound 46 exhibited superior potency than compound 44 both in Brd4 binding assays (K_i = 1.5 nM) and antiproliferation effect on MX-1 cancer cells (EC₅₀ = 13 nM). Compound 46 displayed favorable PK profiles with moderate oral bioavailability (50%) and a fairly good oral half-life (T_{1/2} = 25 h) in humans. Compound 46 achieved a TGI of 99% administered orally Q.D. at a dose of 1 mg kg⁻¹ for 25 days with acceptable tolerability (weight loss ≤10%) in a Kasumi-1 AML mouse xenograft model, more

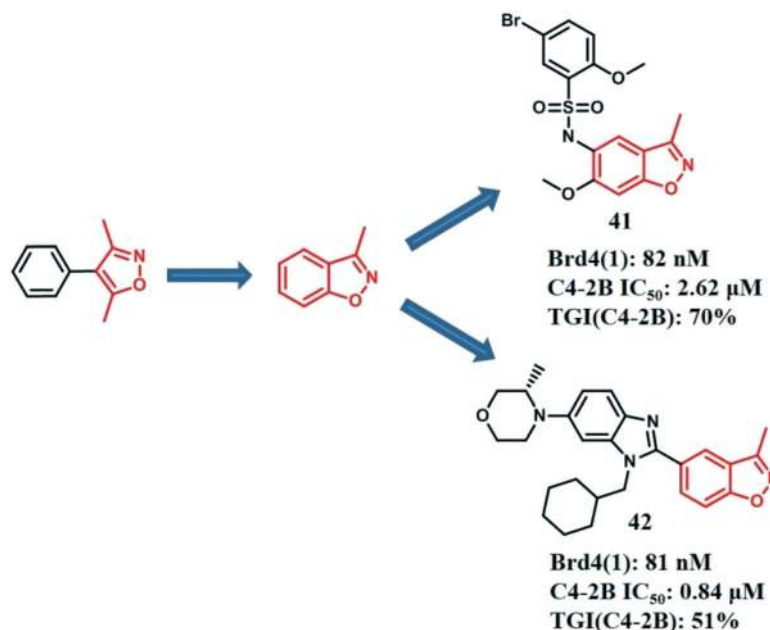


Fig. 7 Reported benzo[*d*]isoxazole-based Brd4 inhibitors.

potent than 5-azacitidine with a TGI of 78% at its maximum tolerated dose. Compound 46 at doses ranging from 1 to 2 mg kg⁻¹ also demonstrated robust *in vivo* antitumor efficacy against both hematologic and solid tumors.⁶¹

A number of other pyridone-based potent Brd4 inhibitors including compounds 47–51 with IC₅₀ values in the low nanomolar level have also been reported in patents (Fig. 8).^{62,63}

AbbVie and Jubilant Biosys filed patents for a number of fused polycyclic pyridones as potent Brd4 inhibitors.^{64,65} Compounds 52–55, representatives of this design, demonstrated Brd4(1) IC₅₀ values in single-digit nanomolar levels, and their good protein potency translated into the cellular assay. Both compounds 52 and 53 demonstrated an EC₅₀ of 16 nM in an MX-1 cellular assay and showed impressive efficacy in mouse tumor xenograft models. Compounds 54 and 55 displayed remarkable MV4-11 antiproliferative activity with EC₅₀ values of 2.0 nM and 8.0 nM, respectively (Fig. 9).

1.1.4 Tetrahydroquinoline-based Brd4 inhibitors. Compounds 56 and 57 were identified as novel *N*-acetyllysine mimetics by Chung and co-workers *via* fragment-based screenings and were considered as appealing starting points to exploit novel Brd4 inhibitors due to their chemical tractability.⁶⁶

GlaxoSmithKline developed a tetrahydroquinoline-based Brd4 inhibitor, compound 58 (I-BET726), on the basis of

N-acetyllysine mimetics compound 56.⁶⁷ Compound 58 showed high binding affinities to Brd4 with *K_d* values of 23 and 4.4 nM, confirmed by surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC), respectively. Compound 58 demonstrated a high degree of selectivity against non-BET bromodomain-containing proteins as well as nonbromodomain targets. Moreover, it achieved a moderate elimination half-life and excellent oral bioavailability. Compound 58 strongly inhibited the proliferation of a panel of neuroblastoma cell lines with a median IC₅₀ value of 75 nM. Oral administration of compound 58 to mouse xenograft models of human CHP-212 neuroblastoma resulted in down-regulation of BCL2 and MYCN and a TGI of 82%.⁶⁸

Novel tetrahydroquinoline-based Brd4 inhibitor compound 59 has been patented by GlaxoSmithKline and showed marked inhibition against Brd4(2) with an IC₅₀ value of less than 100 nM; however, it showed weak inhibition against Brd4(1).⁶⁹ Compounds 60 and 61, patented by Forma Therapeutics⁷⁰ and Bayer,⁷¹ respectively, also displayed potent Brd4 inhibitory activity with IC₅₀ values in the nanomolar range.

Fish *et al.* described the discovery of compound 62 through optimization of the hit compound 57.⁷² Compound 62 displayed an IC₅₀ value of 220 nM against Brd4(1) and inhibited IL-6 production in human blood mononuclear cells

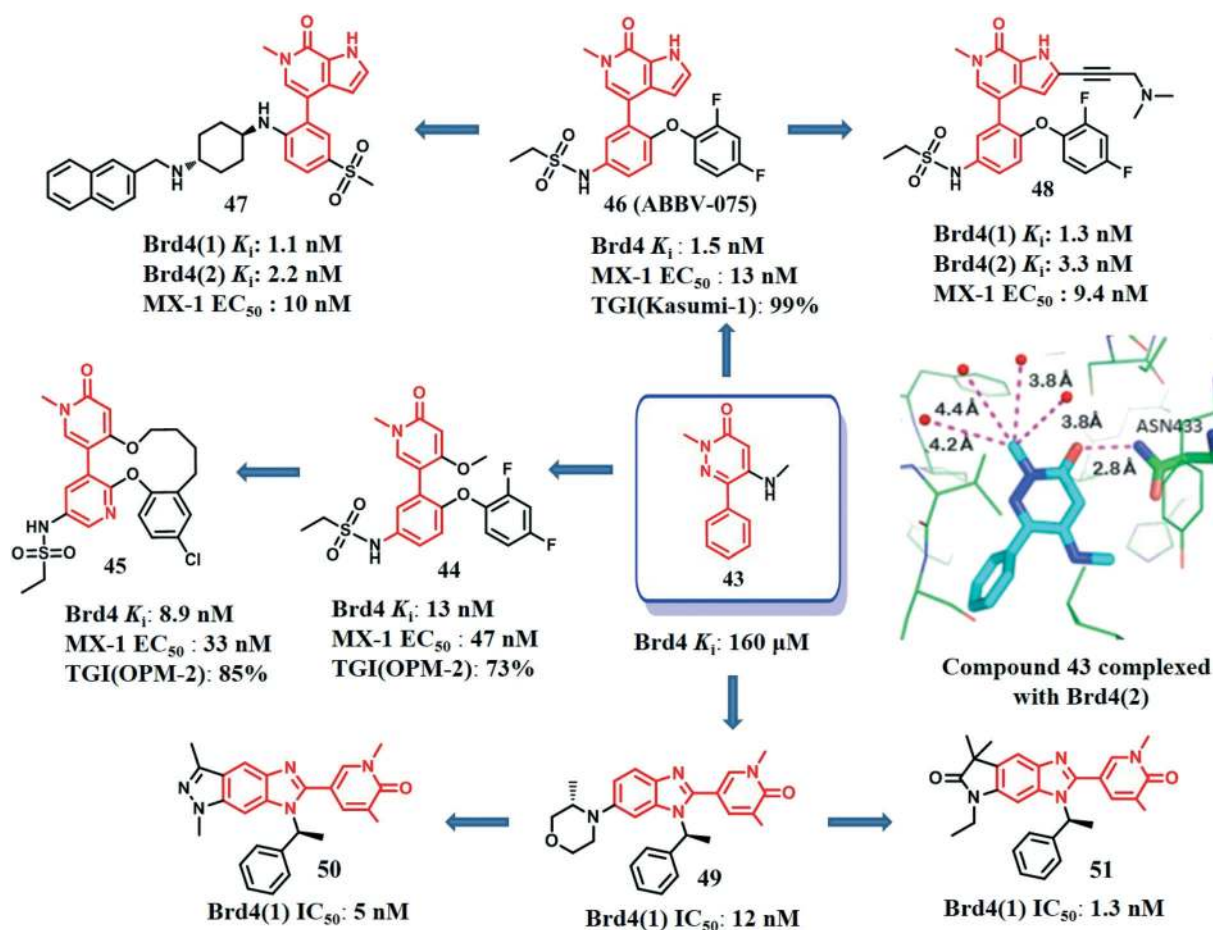


Fig. 8 Reported pyridone-based Brd4 inhibitors.

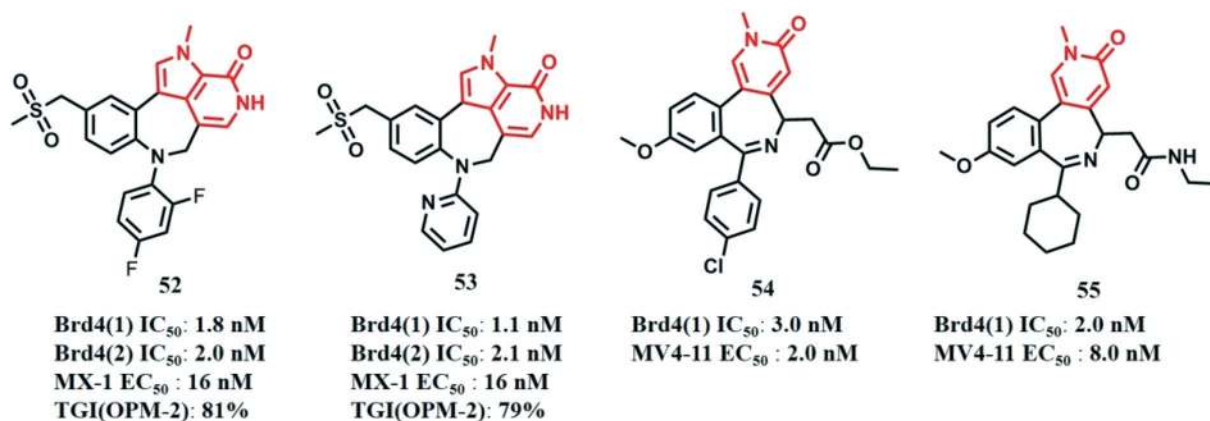


Fig. 9 Reported fused polycyclic pyridone-based Brd4 inhibitors.

stimulated by LPS with an EC₅₀ value of 1.89 μM. Compound 62 showed thermal shifts of 2.1–6.5 °C against BET proteins, while it showed thermal shifts of less than 1 °C against three other tested bromodomain-containing proteins, indicating good selectivity for the BET bromodomains (Fig. 10).

1.1.5 Triazolopyrazine-based Brd4 inhibitors. A Boehringer Ingelheim GmbH patent described Brd4 inhibitors predominantly extended on the triazolopyrazine scaffold; the representative compounds included compounds 63–66, which were recently highlighted in *ACS Med. Chem. Lett.*⁷³ Compounds 63–66 displayed potent Brd4(1) inhibition with remarkable IC₅₀ values in the single-digit nanomolar range (Fig. 11).⁷⁴

Compound 67 and compounds 68–69 were obtained and patented by using a triazolopyridazine core⁷⁵ or a triazolopyridine core⁷⁶ as a bioisostere of triazolopyrazine, respectively. Compounds 67–69 showed low nanomolar potencies in inhibition of Brd4 and MV4-11 cancer cell growth.

1.1.6 4-Acyl pyrrole-based Brd4 inhibitors. Lucas *et al.* performed a high-throughput virtual screening campaign on Brd4 to identify novel inhibitors. This screening led to 22 hits whose binding affinity towards Brd4(1) was determined by ITC assay, identifying compound 70 bearing a 4-acyl pyrrole motif as the most potent hit against Brd4(1) with a K_d value of 237 nM.⁷⁷ Among 56 cell lines tested, compound 70 showed strong

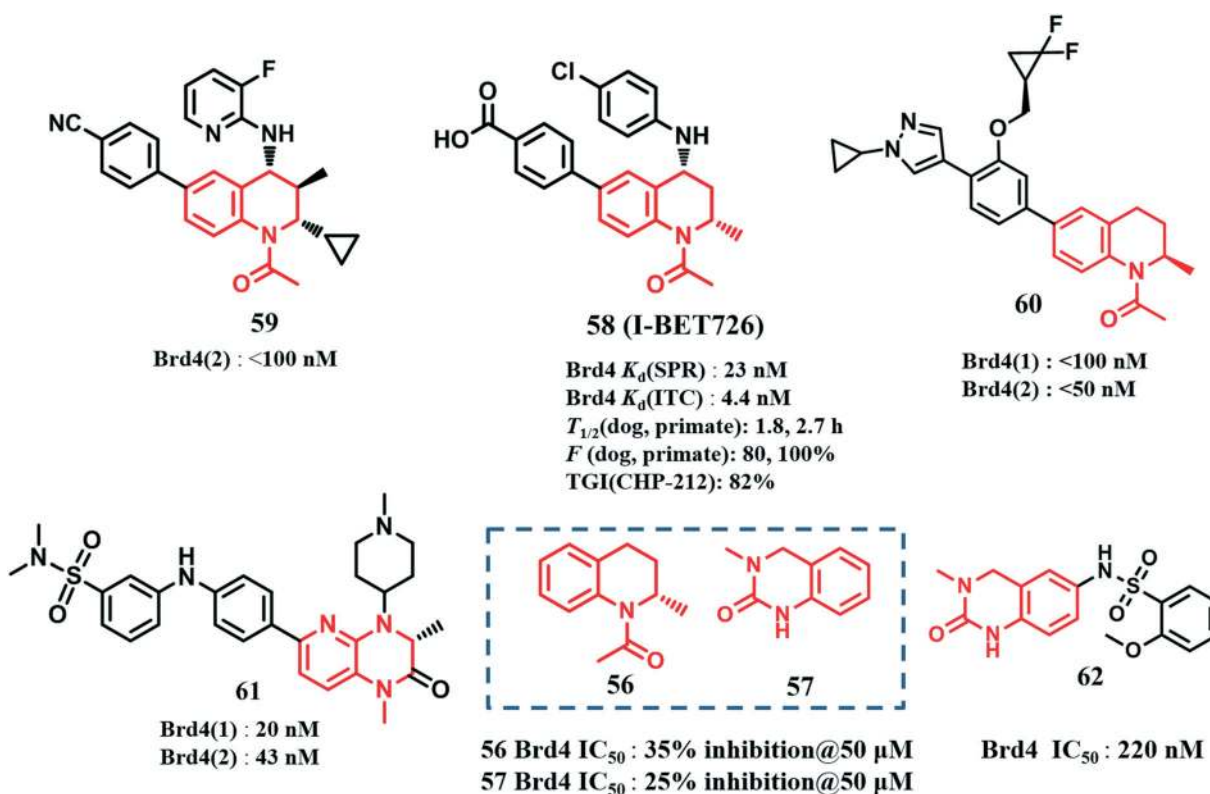


Fig. 10 Reported tetrahydroquinoline-based Brd4 inhibitors.

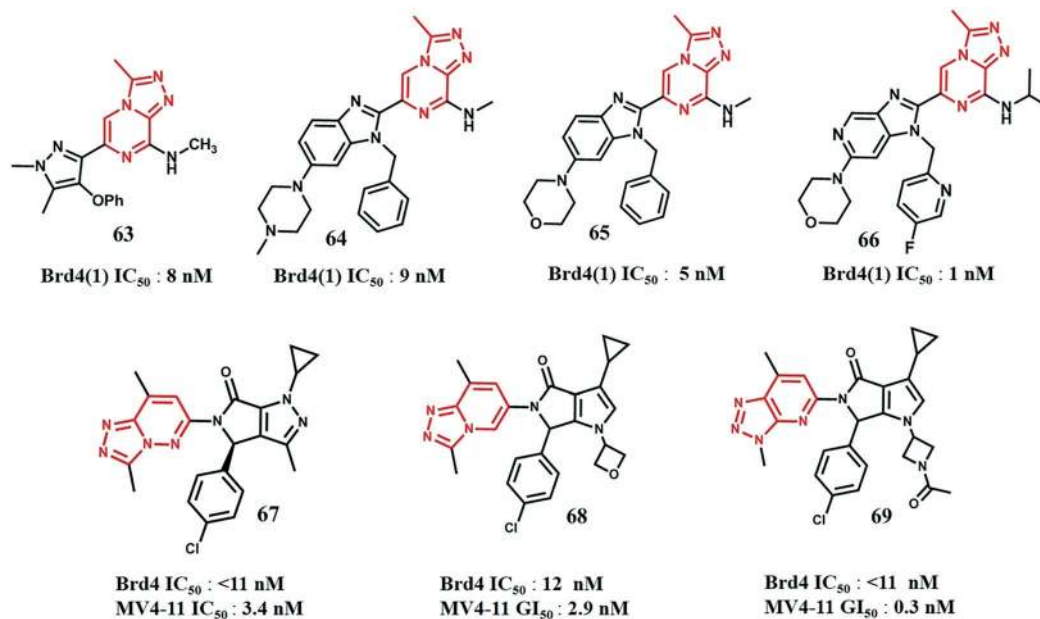


Fig. 11 Reported triazolopyrazine-based Brd4 inhibitors.

inhibitory potency and selectivity towards leukemia cells, with a maximum 65% growth inhibition at 10 μ M against HL-60 cancer cells. A structure-guided modification of compound 70 performed based on the cocrystal structure of Brd4(1)/70 led to Brd4 inhibitors 71 and 72 with K_d values of 0.46 and 0.81 μ M, respectively; however, both were less potent than compound 70 (Fig. 12).⁷⁸

AbbVie published a patent application describing 4-acyl pyrrole-based Brd4 inhibitors, as represented by compound 73, which was potent both biochemically [Brd4(1) K_i = 38 nM, Brd4(2) K_i = 225 nM] and in the MX-1 cell line (EC₅₀ = 433 nM).⁷⁹ Compound 74, patented also by AbbVie, exhibited K_i values of 15 nM and 43 nM against Brd4(1) and Brd4(2), re-

spectively. Compound 74 was potently active against the MX-1 cell line with an EC₅₀ value of 160 nM, which translated into *in vivo* efficacy with a TGI of 80% at a dose of 100 mg kg⁻¹ in an MX-1 mouse xenograft model.⁸⁰

1.1.7 2-Thiazolidinone-based Brd4 inhibitors. Shen's group described compound 76 as a novel Brd4 inhibitor, which was synthesized based on the novel *N*-acetyllysine mimic compound 75 featured with a 2-thiazolidinone core.⁸¹ Compound 76 displayed Brd4(1) inhibition with an IC₅₀ of 4.1 μ M and had good metabolic stability, but showed weak proliferation inhibition activity in HT-29 cancer cells with a GI₅₀ of 47.8 μ M. Subsequent optimization by replacing the sulfonylamino phenyl substitution of 76 with an amino sulfonyl

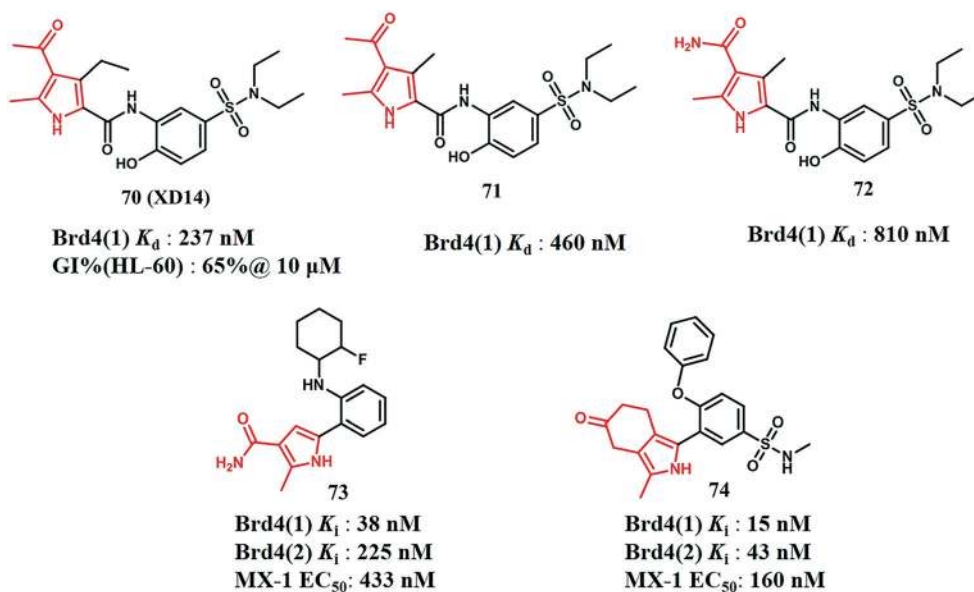


Fig. 12 Reported 4-acyl pyrrole-based Brd4 inhibitors.

phenyl group gave compound 77 with an IC_{50} of 140 nM, which was about 30 times more potent than compound 76.⁸¹ Compound 77 was found to exhibit potent activity against the proliferation of two leukemia cell lines and one colon cancer cell line with IC_{50} values ranging from 184 to 860 nM (Fig. 13).

1.1.8 Other reported Brd4 inhibitors. Other selected examples of Brd4 inhibitors are listed in Fig. 14. Xu and coworker reported the discovery of the benzo[*cd*]indol-2(1*H*)-one scaffold as a new class of BET bromodomain inhibitors *via* a structure-based virtual screening approach and subsequent extensive chemical optimization.⁸² Among them, representative compound 78 showed robust binding affinity to Brd4(1) with a K_d value of 137 nM and excellent selectivity over other non-BET bromodomain-containing proteins in the ITC assay. In MV4-11 leukemia cells and HL-60 cancer cells, compound 78 exhibited reasonable antiproliferation effects with IC_{50} values of 1.30 μ M and 2.99 μ M, respectively. Oral administration with compound 78 in rats demonstrated good pharmacokinetic profiles with high oral bioavailability (76.8%) and a moderate half-life ($T_{1/2}$ = 3.95 h).

William's group disclosed dihydropyridopyrimidine derivative 79 as a novel Brd4 inhibitor, which was readily generated in a single step from commercially available starting materials.⁸³ Compound 79 had a K_i of 110 nM for Brd4(1) and strongly inhibited the growth of MM1.S cells with an IC_{50} value of 0.46 μ M. Compound 79 also demonstrated potent binding affinity to BrdT(1) with a K_i of 200 nM. Compound 79 was identified as the best Brd4 inhibitor (IC_{50} = 0.43 μ M) with good selectivity over other BET family proteins.

Compound 80 markedly inhibited Brd4 and its downstream target c-Myc, induced ATG5-dependent autophagy *via* blocking Brd4-AMPK (AMP-activated protein kinase) interaction and displayed potent antiproliferative activity in breast cancer cell lines (MCF-7, IC_{50} = 1.62 μ M; MDA-MB-231, IC_{50} = 3.27 μ M). Moreover, in *in vivo* efficacy evaluations of MCF-7 and MDA-MB-231 xenograft models, compound 80 showed substantial antitumor activity with a TGI of 80% and 76% after intragastric administration of 100 mg kg^{-1} , respectively, without causing significant loss of body weight and toxicity.⁸⁴

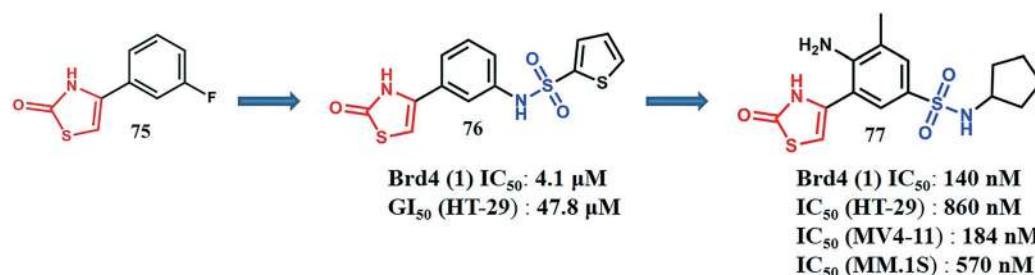


Fig. 13 Reported 2-thiazolidinone-based Brd4 inhibitors.

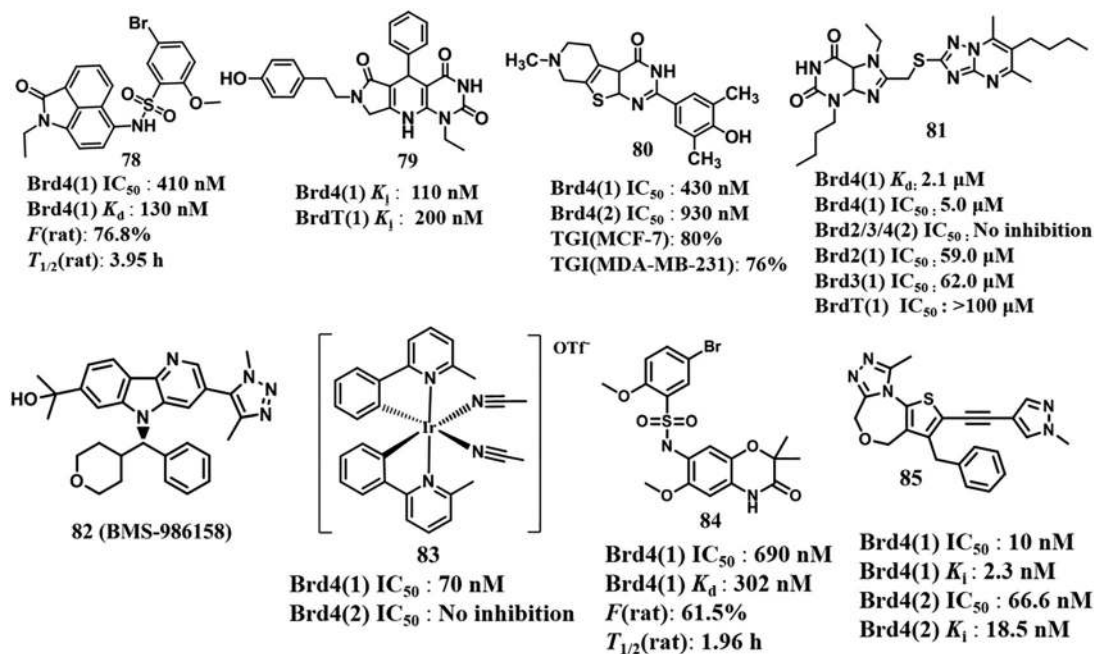


Fig. 14 Other reported Brd4 inhibitors.

Through a midthroughput screen, a xanthine derivative compound **81** was identified as a Brd4 inhibitor with a K_d of 2.1 μM determined by ITC. Despite compound **81** showing weak cellular activity against Jurkat T cells with an EC_{50} of 27 μM , impressive selectivity for Brd4(1) ($\text{IC}_{50} = 5.0 \mu\text{M}$) over Brd2/3(1) ($\text{IC}_{50} > 50 \mu\text{M}$) and BrdT(1) ($\text{IC}_{50} > 100 \mu\text{M}$) was observed; more unexpectedly, compound **81** showed no detectable inhibition against the BD2 counterparts of all BET proteins tested.⁸⁵

Compound **82** (BMS-986158) is a novel Brd4 small-molecule inhibitor developed by Bristol-Myers Squibb and is currently being tested as monotherapy or in combination with nivolumab in subjects with selected advanced solid tumors or hematologic malignancies. However, to date, no clinical data on BMS-986158 have been published in peer-reviewed journals.⁸⁶

Zhong *et al.* identified an iridium(III) complex as the first metal-based, irreversible Brd4 inhibitor, compound **83**.⁸⁷ Compound **83** strongly inhibited Brd4(1) with an IC_{50} value of 70 nM in a TR-FRET assay, while it showed no significant inhibition against Brd4(2). ChIP analysis and immunoblotting analysis revealed that compound **83** could disrupt the binding of Brd4 to the c-Myc and Bcl-2 promoters and reduced the expression of c-Myc and Bcl-2 in A375 and A2058 cancer cells. In a xenograft mouse model of human A375 melanoma cells, compound **83** significantly repressed tumor growth (a 40% reduction of tumor volume) without causing visible toxicity at a dose of 100 mg kg^{-1} once daily for 16 days.

Xu's group designed and synthesized a series of 2,2-dimethyl-2H-benzo[*b*][1,4]oxazin-3(4H)-one derivatives and evaluated their Brd4 inhibitory activities, obtaining several compounds as potent Brd4 inhibitors with IC_{50} values in the nanomolar range.⁸⁸ The representative compound **84** potently inhibited Brd4(1) with an IC_{50} value of 690 nM and a K_d value

of 302 nM determined by an ITC experiment. Compound **84** showed good inhibition against a panel of prostate cancer cell lines, including C4-2B, LNCaP, and 22Rv1, with IC_{50} values in the range of 3.23–4.51 μM , and potently inhibited 22Rv1 cancer cell colony formation in a dose-dependent manner. Oral dosing with compound **84** in rats demonstrated reasonable pharmacokinetic properties with a $T_{1/2}$ value of 1.96 h and an oral bioavailability of 61.5%.

Wang's group described a series of [1,4]oxazepine derivatives as a new class of Brd4 inhibitors. The most potent, compound **85**, bound to Brd4(1) with a K_i value of 2.3 nM and an IC_{50} value of 10 nM and was more potent than OTX015. Consistent with its high binding affinities to Brd4, compound **85** was an effective inhibitor of cell growth, with IC_{50} values of 55.8, 207, and 173 nM in the MV4-11, MOLM-13, and RS4-11 cell lines, respectively.⁸⁹

1.2 Bivalent Brd4 inhibitors

Waring and colleagues demonstrated a series of triazolopyridazine-containing compounds, which were capable of binding both Brds in a single Brd4 protein simultaneously.⁹⁰ The first bivalent Brd4 inhibitor, compound **87**, was accidentally generated based on the structure optimization of AZD3514 (**86**), an androgen receptor (AR) modulator (Fig. 15).^{91,92} Despite its increased AR down-regulation potency, however, compound **87** induced estrogen receptor- α (ER α) down-regulation, indicating that the direct target of compound **87** might not be AR. Considering the structural similarity between compound **87** and Brd4 inhibitor I-BET762, a panel of Brd inhibition assays were performed. Compound **87** displayed potent inhibition against Brd4(1) and Brd4(2) with pK_d values of 7.2 and 6.1, respectively. Compound **87** suppressed c-Myc levels, inhibited cell growth and induced apoptosis more effectively in several Brd4-sensitive

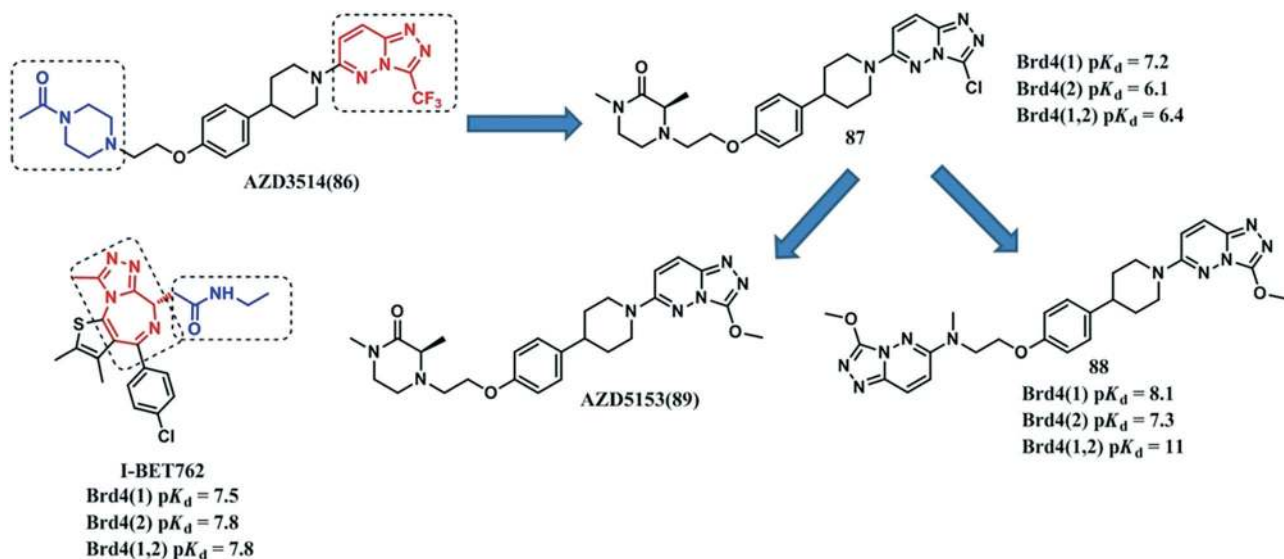


Fig. 15 Discovery and development of bivalent Brd4 inhibitor **89**.

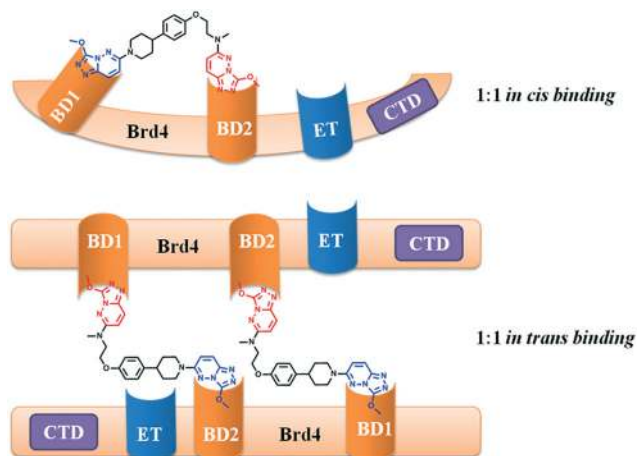


Fig. 16 Binding modes for bivalent Brd4 inhibitor 88.

cancer cells than less sensitive cancer cells, consistent with its marked Brd4 inhibition. The cocrystal structure of Brd4(1) with compound 87 revealed that compound 87 could remarkably induce dimerization of two Brd4(1) by spanning both the acetyl-lysine binding pocket *via* the triazolopyridazine motif and the piperazinone group.

Further modification of compound 87 by changing the piperazinone group or chloro-substituted triazolopyridazine with a methoxy-substituted triazolopyridazine, a more potent acetyl-lysine pocket binding scaffold, gave compounds 88 and 89 (AZD5153), respectively.⁹³ Compound 88 was identified as the most potent Brd4 inhibitor reported to date with pK_d values of 8.1 and 7.3 against Brd4(1) and Brd4(2), respectively. It was also found that compound 88 showed few off-target effects on cellular receptors and ion channels, more than 100 of which were tested. The increased Brd4 inhibition potency was reflected in its cellular activity; compound 88 displayed highly potent antiproliferation activity against both MV4-11 and MM.1S cells with a pIC_{50} of 9.5, which was 1000-fold more potent than that of I-BET762. The therapeutic advantages of bivalent Brd4 inhibition by compound 88 was also evidenced by the near-complete cell killing in RS4-11 cancer cells, compared to I-BET762 with no full inhibition at even higher concentrations. Compound 88 was proved to be more significant and sustained in suppressing the c-Myc expression, which might be responsible for its enhanced antiproliferation activity.

Chen's group reported the anticancer effects of AZD5153 against hematologic malignancies. AZD5153 potently inhibited

Brd4 with IC_{50} values of 5 nM against full-length Brd4 and 1.6 μ M against Brd4(1). AZD5153 was more potent than the monovalent inhibitor I-BET762 in down-regulating c-Myc, with a c-Myc protein modulation IC_{50} of 5.4 nM for AZD5153 compared with an IC_{50} of 329 nM for I-BET762. AZD5153 displayed an excellent pharmacokinetic profile and tumor growth inhibition in xenograft studies. In a xenograft model of MV4-11 cancer cells, daily treatment with 1 mg kg^{-1} AZD5153 gave a TGI of 72%, while 5 mg kg^{-1} daily oral doses of AZD5153 led to tumor repression.⁹⁴ AZD5153 is now in a phase I clinical trial by AstraZeneca for treatment of malignant solid tumors and lymphoma.⁹⁵

Compound 88 engaged two Brd4(1)s concurrently in a fashion similar to that observed with compound 87 and reduced a Brd4(1) dimer confirmed by X-ray. A variety of biophysical experiments including NMR spectroscopy, analytical ultracentrifugation, and small-angle X-ray scattering were performed to elucidate the binding mode of 88 with full-length Brd4 in cells. The results showed that compound 88 simultaneously bound both BD1 and BD2 of a single Brd4 in *cis* binding fashion and brought the two Brds into close proximity *via* folding the Brd4 protein (Fig. 16).

Bradner *et al.* described a series of bivalent Brd4 inhibitors by tethering two (+)-JQ1 molecules *via* a variable-length polyethylene glycol linker. Among them, compound MT1 (90) was identified as a more potent Brd4 bivalent inhibitor with improvement of exposure time and PK properties *in vivo* compared to (+)-JQ1 (Fig. 17).⁹⁶ Size-exclusion chromatography (SEC) with bromodomain mutant experiments supported that one MT1 molecule simultaneously inhibited both BD1 and BD2 of Brd4 in *cis* binding mode, as observed with compound 88. In cellular assays, 100 nM MT1 remarkably and rapidly depleted c-Myc protein, up-regulated HEXIM, and showed a 10-fold higher potency in reducing apoptosis in MV4-11 cells than the corresponding monovalent inhibitor, (+)-JQ1. Mouse studies exhibited that a half-equivalence of MT1 significantly delayed leukemia progression in an aggressive disseminated leukemia mouse model (mCherry+, luciferase+ MV4-11) compared to (+)-JQ1. The findings of SEC, ITC and nanomaterial-based proximity assays supported that the improved antitumor activity of MT1 was associated with its ability to dimerize Brds.

2 Brd4 degraders

While Brd4 inhibitors have demonstrated their promising therapeutic potential in a variety of c-Myc-driven malignancies,

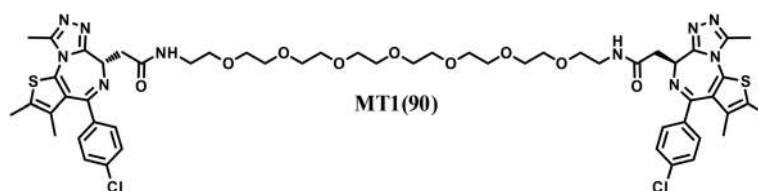


Fig. 17 The structure of bivalent Brd4 inhibitor MT1.

several recent studies showed that Brd4 inhibitors led to significant compensatory accumulation of Brd4 protein in several cancers including Burkitt's lymphoma, lung cancer and prostate cancer, which may account for their inefficient *c-Myc* suppression, modest apoptotic induction and antiproliferative activity.^{97,98} Moreover, drug resistance against triazoloazepine-based Brd4 inhibitors I-BET762 and (+)-JQ1 has been described.⁹⁹

In addition to target protein inhibition, selective induced target protein degradation was emerging as a novel drug discovery strategy.^{100,101} One promising approach to degrade protein is to design proteolysis-targeting chimeras (PROTACs), which has draw extensive attention from medicinal chemists and pharmaceutical firms.^{100,102–105} PROTAC, initially proposed 15 years ago by Deshaies *et al.*,¹⁰⁶ was a hetero-functional bispecific small molecule. It comprises three components: a target protein-specific ligand, an E3 ubiquitin ligase binder and a linker that couples these two functionalities. The PROTACs forms a ternary complex by binding to both the target protein and a component of E3 ubiquitin ligase in a spatially favorable presentation to promote the target protein ubiquitination by the E3 ligases, thereby eliciting its ubiquitination and subsequent proteasomal degradation (Fig. 18).^{107,108}

Theoretically, degradation of oncoproteins by PROTACs exhibited potential advantages over protein inhibition in cancer treatment. Firstly, removal of the entire protein is expected to be more effective compared to inhibiting an individual activity site, which leaves other parts and domains of the proteins still functional; secondly, PROTACs could act catalytically to degrade superstoichiometric amounts of the target protein; thirdly, “undruggable” proteins including transcription factors could be targeted by PROTACs.^{109,110}

PROTACs have recently been used to degrade a variety of proteins including tyrosine kinases,¹¹¹ estrogen receptor α ,¹¹² CDK9,¹¹³ Bcr-Abl^{114,115} and so on. Considering the therapeutic potential of epigenetic regulator Brd4 in cancers and compensatory protein up-regulation often observed upon Brd4 inhibition, several groups sought to utilize PROTACs to degrade

Brd4 to treat cancers. Recently reported Brd4-targeted PROTACs were mainly designed based on thalidomide derivatives (91–93) identified as small ligands of cereblon (CRBN), a component of the CRL4^{CRBN} E3 ligases,¹¹⁶ and VHL-2 (94) and VH-032 (95), two high-affinity ligands of the CRL2^{VHL} E3 complex (Table 2) (Fig. 19).^{117,118}

2.1 CRL4^{CRBN} E3-based Brd4 degraders

CRL4^{CRBN} E3-based PROTACs targeting Brd4 were synthesized by connecting thalidomide derivatives to various Brd4 small-molecule inhibitors *via* different linkers (Fig. 20). The aryl ring of thalidomide derivatives and the carboxyl scaffold on Brd4 inhibitors can tolerate chemical substitution and so were chosen as suitable connecting points for a linker.

Winter *et al.* demonstrated a small-molecule CRL4^{CRBN} E3-based degrader compound 96 (**dBET1**), which consisted of a Brd4 inhibitor (+)-JQ1 linked to a thalidomide derivative, which binds CRBN.¹¹⁹ On treatment with the MV4-11 cell line for 2 hours at a concentration as low as 100 nM, **dBET1** could entirely degrade Brd4 and significantly reduced its transcriptional target *c-Myc*, with minimal off-target degradation. Degradation of Brd4 by **dBET1** induced a more potent and superior inhibitory effect than (+)-JQ1 against MV4-11 cells, DHL4 lymphoma cells and primary blasts from patients with leukemia. The therapeutic opportunity of **dBET1** *in vivo* was evaluated in a murine hind-limb xenograft model of human MV4-11 leukemia cells and an aggressive disseminated leukemia mouse model (mCherry+ MV4-11). In the AML model, daily treatment with **dBET1** significantly attenuated tumor progression and decreased tumor weight in two weeks, together with Brd4 degradation and *c-Myc* down-regulation. Notably, in the disseminated model, **dBET1** treatment caused a greater decrease in leukemic burden in bone marrow than did (+)-JQ1.

Concurrent with Winter's study, Crews *et al.* reported another small-molecule CRL4^{CRBN} E3-based PROTAC ARV-825 (97), which was capable of degrading Brd4 rapidly at picomolar potencies in Burkitt's lymphoma cells.⁹⁷ ARV-825 was synthesized by connecting a small-molecule Brd4 inhibitor (OTX015)

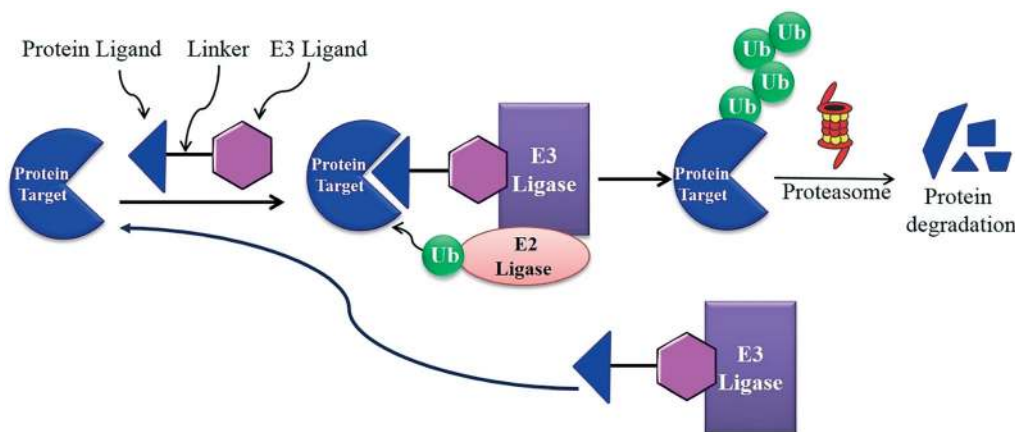


Fig. 18 Mechanism of protein degradation by PROTACs.

Table 2 Overview of small-molecule Brd4 degraders

Comp.	Brd4 inhibitor	E3 ligand	E3 ligase	Cellular activity	<i>In vivo</i> activity	Ref.
96 (dBET1)	(+)-JQ1	Thalidomide	CRL4 ^{CRBN}	Entirely depleted Brd4 in MV4-11 cells at 100 nM with a 2 h treatment	Significantly decreased leukemic burden in bone marrow mCherry+ MV4-11 xenografts model: 63.8 $\mu\text{M kg}^{-1}$, once daily for 19 days	119
97 (ARV-825)	OTX015	Pomalidomide	CRL4 ^{CRBN}	Remarkably reduced the levels of Brd4 and c-Myc with a 2 h treatment Namalwa cells: DC ₅₀ < 1 nM Ramos cells: DC ₅₀ < 1 nM Efficient and sustained depletion of Brd4 with an 18 h treatment Mino cells: 500 nM Z138 cells: 500 nM Induction of apoptosis IC ₅₀ (mino) = 16 nM IC ₅₀ (ibrutinib-resistant Z138) = 327 nM IC ₅₀ (sAML SET2) = 14.5 nM IC ₅₀ (sAML UKE1) = 256 nM	Not reported	97, 120, 121
98 (BETd-246)	HJB97	Lenalidomide	CRL4 ^{CRBN}	Completely reduced the levels of Brd4 with a 3 h treatment MDA-MB-453: 30 nM Cell growth inhibition IC ₅₀ (MDA-MB-453) < 10 nM IC ₉₀ (MDA-MB-453) < 100 nM	TGI: 85% MDA-MB-453 xenograft model: i.v., 5 mg kg^{-1} , 3 times per week for 3 weeks	122
99 (BETd-260)	HJB97	Lenalidomide	CRL4 ^{CRBN}	Remarkably reduced the levels of Brd4 and c-Myc with a 24 h treatment RS4-11: < 30 pM, 0.1 nM Cell growth inhibition IC ₅₀ (MOLM-13) = 2.3 nM IC ₅₀ (RS4-11) = 51 pM	Rapid tumor regression with a maximum of >90% tumor regression RS4-11 xenograft model: i.v., 5 mg kg^{-1} , three times a week for 3 weeks	122, 123
100 (QCA570)	QCA276	Lenalidomide	CRL4 ^{CRBN}	Remarkably reduced the levels of Brd4 and c-Myc with a 3 h treatment RS4-11: < 10 pM MV4-11: < 10 pM, 30 pM Cell growth inhibition IC ₅₀ (MV4-11) = 8.3 pM IC ₅₀ (MOLM-13) = 63 pM IC ₅₀ (RS4-11) = 32 pM	Complete and long-lasting tumor regression RS4-11 xenograft model: i.v., 1, 2.5, and 5 mg kg^{-1} , three times a week for 3 weeks MV4-11 xenograft model: i.v., 5 mg kg^{-1} , three times per week for 2 weeks	89
101 (ARV-771)	OTX015	VHL-2	CRL2 ^{VHL}	Potently reduced the levels of Brd4 with a 16 h treatment 22Rv1: DC ₅₀ < 5 nM VCaP: DC ₅₀ < 5 nM LnCaP95: DC ₅₀ < 5 nM Efficient and sustained depletion of Brd4 with an 18 h treatment Mino cells: 500 nM Z138 cells: 500 nM Induction of apoptosis IC ₅₀ (mino) = 17 nM IC ₅₀ (ibrutinib-resistant Z138) = 142 nM	Tumor regression 22Rv1 xenograft model: s.c., 30 mg kg^{-1} , once daily for 15 days TGI: 60% VCaP xenograft model: s.c., 30 mg kg^{-1} , one time per 3 days for 15 days Significantly improve the median and overall survival of the NSG mice MCL Z138/Luc xenografts in NSG mice: s.c., 30 mg kg^{-1} , daily \times 5 days per week for 3 weeks	124, 125
102 (MZ1)	(+)-JQ1	VH-032	CRL2 ^{VHL}	Completely depleted Brd4 with a 4 h treatment HeLa cells: 1 μM U2OS: 5 μM	Not reported	126
104 (AT1)	(+)-JQ1	90 (VH-032)	CRL2 ^{VHL}	Remarkable Brd4-selective depletion in HeLa cells with a 24 h treatment at 1 μM Potently reduced the levels of Brd4 in HeLa cells with a 24 h treatment	Not reported	107

Table 2 (continued)

Comp.	Brd4 inhibitor	E3 ligand	E3 ligase	Cellular activity	<i>In vivo</i> activity	Ref.
106 (MZP-54)	I-BET726	90 (VH-032)	CRL2 ^{VHL}	pDC ₅₀ (Brd4 short) = 8.0 pDC ₅₀ (Brd4 long) = 7.6 Cell growth inhibition pEC ₅₀ (MV4-11) = 7.31 pEC ₅₀ (HeLa) = 6.57	Not reported	127

to pomalidomide *via* a flexible polyethylene glycol linker. Treatment of Burkitt's lymphoma cells with ARV-825 led to 50% Brd4 protein degradation within 2 hours, with a DC₅₀ (50% of maximum degradation) below 1 nM. Given that the K_d values of OTX015 and pomalidomide to their respective targets, Brd4 and cereblon, were 10 nM and 3 μM, respectively, ARV-825 showed sub-stoichiometric characteristics in degrading Brd4.¹²⁰ In comparison to the Brd4 inhibitors (+)-JQ1 and OTX015, ARV-825 provided a more prolonged and pronounced suppression of c-Myc levels even at lower concentrations and showed no Brd4 accumulation which was associated with Brd4 inhibition. This sustained suppression of c-Myc translated into superior antiproliferative and apoptotic effects of the ARV-825 against Burkitt's lymphoma cells compared with the Brd4 inhibitors. They also found that ARV-825 was more profound and longer-lasting than OTX015 in depleting Brd4, c-Myc, CDK4/6, JAK2, pSTAT3/5, PIM1 and Bcl-xL in cultured and patient-derived CD34⁺ post-MPN sAML cells, including those expressing JAK2-V617F and mutant TP53, consistent with higher ARV-825-induced apoptosis and lethality in sAML cells.¹²¹

BETd-246 (98) was developed by Wang's group using an azacarbazole-containing Brd4 inhibitor HJB97 coupled to lenalidomide *via* a polyethylene glycol linker.¹²² The majority of Brd4 was depleted after treatment with BETd-246 for three hours at 10 to 30 nM in several triple-negative breast cell lines. BETd-246 strongly suppressed the growth of 9 TNBC cell lines with IC₅₀ < 10 nM, and was 50-fold more potent than HJB97. In both Washington Human in Mouse (WHIM) and MDA-MB-453 xenograft models, BETd-246 achieved significant antitumor activity and induced partial tumor regression at well-tolerated dose schedules. However, BETd-246

showed very limited or no antitumor activity in MDA-M-231 and MDA-MB-468 xenograft models, respectively, due to limited drug exposure in both models.

BETd-260 (99) was generated by further structure optimization of BETd-246.¹²³ BETd-260 showed a more potent antiproliferative activity than BETd-246 in TNBC cells, and more importantly, BETd-260 exhibited a much higher drug exposure in both MDA-MB-231 and MDA-MB-468 xenograft models and exerted a much stronger antitumor activity than BETd-246 without inducing observable toxic effects.¹²² BETd-260 was capable of effectively reducing the level of Brd4 protein at concentrations as low as 30 pM with a 24 hour treatment in the RS4-11 leukemia cell line, accompanied by strong down-regulation of c-Myc protein, while HJB97 showed no effect on the level of Brd4 proteins even at 1 μM. BETd-260 significantly inhibited RS4-11 cell line and MOLM-13 cell line proliferation with IC₅₀ values of 51 pM and 2.3 nM, respectively. *In vivo*, treatment of the RS4-11 xenograft model with BETd-260 induced rapid tumor regression with a maximum of >90% regression observed.

More recently, Wang's group reported an exceptionally potent small-molecule BET degrader QCA570 (100), which was synthesized using a novel [1,4]oxazepine-based BET inhibitor developed by Wang's group and known as cereblon ligand.⁸⁹ After 3 h of treatment, QCA570 was capable of effectively reducing the levels of Brd4 and c-Myc at concentrations as low as 10 pM in the RS4-11 cell line and 30 pM in the MV4-11 cell line, while the corresponding Brd4 inhibitor failed to abate the levels of Brd4 and c-Myc at a concentration of 10 μM. Consistent with its extraordinary ability to suppress Brd4, QCA570 showed significant potency in inhibition of cell growth in MV4-11, MOLM-13, and RS4-11 cell lines with IC₅₀ values of 8.3, 62, and 32 pM, respectively. A direct comparison of cell growth inhibitory potency in these three leukemia cell lines between QCA570 and previously published Brd4 degraders including dBET1, ARV-825 and ARV-771 was performed. The results demonstrated that QCA570 was the most potent and efficacious Brd4 degrader to date. Significantly, in both the MV4-11 and RS4-11 acute leukemia xenograft models, QCA570 achieved complete and long-lasting tumor regression at 5 mg kg⁻¹ without apparent toxicity (Fig. 21).

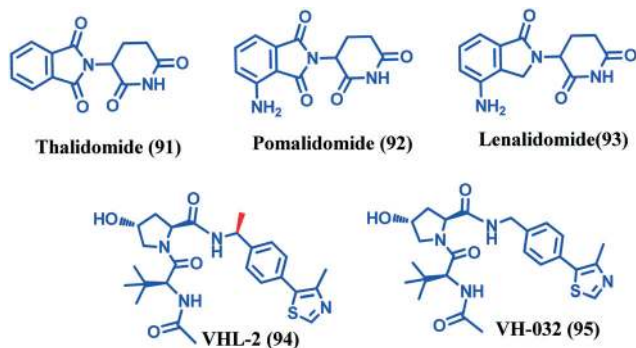


Fig. 19 Reported CRL4^{CRBN} E3 and CRL2^{VHL} E3 small-molecule ligands.

2.2 CRL2^{VHL} E3-based Brd4 degraders

Inspection of VHL and its ligand crystal structures showed that the methyl group of the terminal acetyl groups in compounds

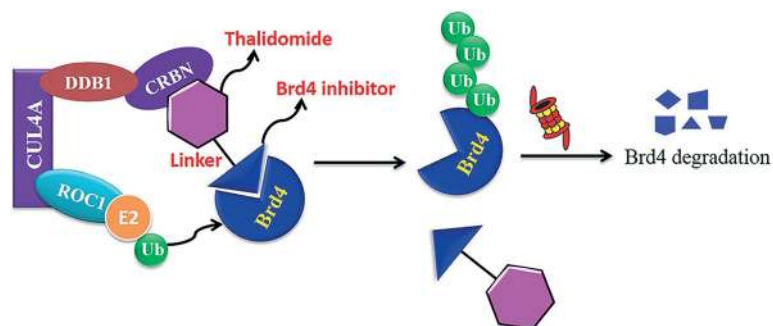


Fig. 20 Mechanism of Brd4 degradation by CRL4^{CRBN} E3-based Brd4 degraders.

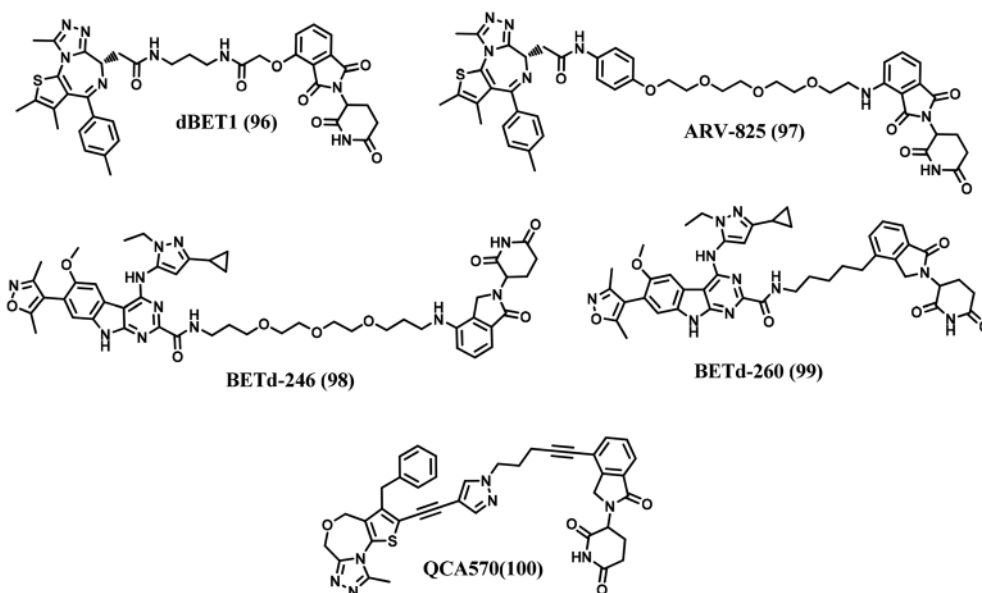


Fig. 21 Reported Brd4 degraders based on CRL4^{CRBN} E3 ligase.

VHL-2 and VH-032 was solvent exposed, indicating that it could provide a suitable connecting point for a linker. By conjugating the terminal acetyl groups of VHL-2 or VH-032 with various Brd4 inhibitors through different linkers, CRL2^{VHL} E3-based Brd4 degraders were obtained (Fig. 22).

ARV-771 (101) is a VHL-based PROTAC using OTX015 for the BET inhibitor portion and VHL-2 as a ligand for the VHL

E3 ligase.¹²⁴ ARV-771 specifically degraded Brd4 as well as Brd2 and Brd3 with a DC₅₀ of <5 nM and led to the depletion of c-Myc protein with an IC₅₀ < 1 nM in several cellular models of castration-resistant prostate cancer. In addition, despite possessing *K_d* values comparable with those of the (+)-JQ1, ARV-771 demonstrated a more than 10 times higher efficacy in decreasing c-Myc levels. This strongly supports

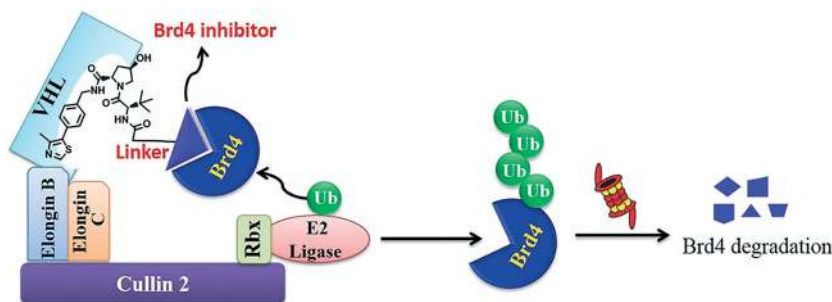


Fig. 22 Mechanism of Brd4 degradation by CRL2^{VHL} E3-based Brd4 degraders.

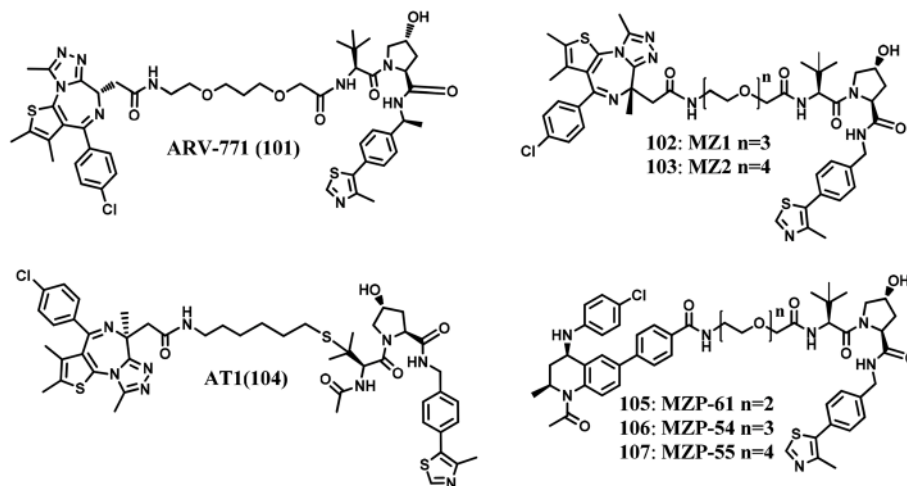


Fig. 23 Reported Brd4 degraders based on CRL2^{VHL} E3 ligase.

that ARV-771 behaved catalytically in degrading Brd4, consistent with previous findings by Winter *et al.* and Crews *et al.*^{119,120} ARV-771 showed significant antiproliferative activity towards prostate cancer cells with 10- to 500-fold higher potency than the inhibitors (+)-JQ1 or OTX015, in line with its robust effects on apoptotic induction. *In vivo* experiments indicated that ARV-771, rather than inhibitor OTX015, effectively attenuated Brd4 and c-Myc levels and suppressed tumor growth and, more notably, induced partial tumor regression in a 22Rv1 xenograft model. ARV-771 also showed superior tumor suppression and induced greater survival improvement than OTX015 in immune-depleted mice engrafted with ibrutinib-resistant MCL cells.¹²⁵

Ciulli and colleagues developed several PROTACs using (+)-JQ1 and VH-032, a high-affinity ligand that had been designed against the VHL E3 ligase.¹²⁶ Treatment with MZ1 (102), the most potent compound, for 24 h at a concentration of 1 μ M completely depleted Brd4 proteins in HeLa cells, and no protein could be detected even after 48 h, indicating that MZ1 was a powerful and long-lasting Brd4 degrader. Interestingly, despite (+)-JQ1 showing no selectivity for individual BET family members and MZ1 having comparable affinities to BET bromodomains, MZ1 could induce potent and preferential depletion of Brd4 over its homologues Brd2 and Brd3 at suitable concentrations in both HeLa cells and U2OS osteosarcoma cells, which partly accounted for the different protein depletion profile and pharmacological response between MZ1 and (+)-JQ1.

Recently, the crystal structure of the VHL E3–MZ1–Brd4 ternary complex and its impact on target degradation selectivity was elucidated by Ciulli *et al.* According to their work, AT1 (103) was synthesized by attaching (+)-JQ1 to the *tert*-Leu group of VHL ligand *via* a short linker, which was different from other VHL-based PROTACs. Treatment with 1 μ M AT1 for 24 h in all tested cancer cells depleted most of the Brd4, while the decrease of Brd2 and Brd3 as well as other 5671 detected proteins was negligible. This result suggested that AT1 exhibited a more remarkable selectivity in depleting Brd4 than MZ1.¹⁰⁷

Most recently, Ciulli's group has reported three novel VHL-recruiting PROTACs, MZP-61 (105), MZP-54 (106) and MZP-55 (107), which were derived from a potent tetrahydroquinoline-based Brd4 inhibitor, I-BET726.¹²⁷ MZP-54 proved to be the most powerful degrader, which was capable of inducing marked depletion of both BET and c-Myc proteins concentration-dependently in HeLa cells with a certain degree of selectivity for Brd4 and Brd3 over Brd2. However, despite I-BET726 ($K_d = 4$ nM) being a more potent Brd4 inhibitor than (+)-JQ1 ($K_d = 100$ nM), a direct comparison showed that MZP-54 was a less powerful Brd4 degrader than MZ1 due to negative cooperativities of ternary complex formation confirmed by ITC assay. Given being negatively cooperative, MZP-54 still effectively degraded Brd4 at nanomolar concentrations and showed remarkable antiproliferative activity in two tested cell lines, suggesting that substoichiometric catalytic degradation was powerful (Fig. 23).

Conclusion

Over the past decade, researchers have identified the cellular functions of BET proteins and their important roles in the development of many malignancies and other diseases including inflammation, HIV infection, and cardiovascular diseases. Among them, Brd4 is the most extensively studied member. Brd4 inhibitors with various scaffolds are being explored as therapeutic agents in numerous hematopoietic and solid tumor types, and some of them have entered into human clinical trials. Despite the exciting results of preliminary clinical trials using Brd4 inhibitors in malignancies, there are issues to resolve. Most of the reported Brd4 inhibitors bind non-selectively to BD1 and BD2 and also show low selectivity for individual BET family members. BET family members have different downstream gene regulatory profiles, and pan-BET inhibition makes an impact on numerous transcriptional pathways and leads to side effects, which has been observed in clinical trials of compound OTX015.¹²⁸ Thus, significant efforts are urgently needed to develop Brd4 inhibitors

with high specificity and affinity. Moreover, drug resistance against triazoloazepine Brd4 inhibitors has been reported.⁹⁹ Considering the dominant position of triazoloazepine-based Brd4 inhibitors in clinical trials, novel Brd4 inhibitors with different chemical chemotypes deserve to be explored to address this issue as well as to elucidate the resistance mechanisms. Bivalent Brd4 inhibitors show profoundly enhanced potency than the parent monovalent inhibitor and may provide a new strategy for overcoming drug resistance. However, the bivalent Brd4 inhibitors reported now are mainly achieved by linking two monovalent inhibitors with a flexible linker, causing a large molecular weight and compromised drug-likeness; thus, there exists a large chemical space to develop structurally diverse bivalent Brd4 inhibitors.

In addition to functional inhibition by small molecules, Brd4 has been successfully targeted for degradation using PROTACs. Several PROTACs selectively and stoichiometrically degraded Brd4 and demonstrated a more potent antitumor activity than the corresponding Brd4 inhibitors. Albeit PROTAC-induced Brd4 degradation has achieved impressive efficacy in several mouse models of cancer, the clinical potential of this technology remains to be explored. Challenges, including unfavorable physicochemical properties and low bioavailability owing to their relatively large molecular mass and scarce small E3 ligands, must be overcome in order to advance PROTACs into the clinic.

Overall, both traditional small-molecule Brd4 inhibitors and newly-developed Brd4 degraders have shown promising results in various human cancers. There is great hope that with sustained efforts on the drug discovery of Brd4, novel anticancer drugs targeting Brd4 will be achieved in the near future.

List of abbreviations

Brd4(1)	Bromodomain 1 of Brd4
Brd4(2)	Bromodomain 2 of Brd4
IC ₅₀	Half-maximal inhibitory concentration
EC ₅₀	Half-maximal effective concentration
GI ₅₀	Half-maximal growth inhibitory concentration
TGI	Tumor growth inhibition
K _d	Dissociation constant
K _i	Inhibition constant
T _{1/2}	Elimination half-life
F	Bioavailability
Q.D.	Once a day

Conflicts of interest

There are no conflicts of interest to declare.

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